Journal of Chromatography, 565 (1991) 1–26
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5760

Review

Determination of L-3,4-dihydroxyphenylalanine in biological fluids and tissues

NIL DIZDAR, ANNEMARIE HENRIKSSON and BERTIL KÅGEDAL*

Departments of Neurology and Clinical Chemistry, University Hospital, S-581 85 Linköping (Sweden)

(First received August 28th, 1990; revised manuscript received December 3rd, 1990)

CONTENTS

0378-4347/91/$03.50

LIST OF ABBREVIATIONS

AADC	Aromatic amino acid decarboxylase
CD	Carbidopa
COMT	Catechol-O-methyl transferase
CSF	Cerebrospinal fluid
C.V.	Coefficient of variation
DA	Dopamine
DHBA	3,4-Dihydroxybenzylamine
DOMA	3,4-Dihydroxymandelic acid
DOPA	3,4-Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenylacetic acid
DOPEG	3,4-Dihydroxyphenylethylene glycol
DOPET	3,4-Dihydroxyphenylethanol
E	Epinephrine
ECD	Electron-capture detection
ED	Electrochemical detection
ECNI	Electron-capture negative-ion
EDTA	Ethylenediaminetetraacetate
GC	Gas chromatography
HPLC	High-performance liquid chromatography
αMD	α-Methyldopamine
MS	Mass spectrometry
NE	Norepinephrine
SAM	S-Adenosylmethionine
TFA	Trifluoroacetyl
TH	Tyrosine hydroxylase
TLC	Thin-layer chromatography

1. INTRODUCTION

L-3,4-Dihydroxyphenylalanine (DOPA) is a direct precursor amino acid of dopamine, which is important for dopaminergic neuron transmission [1,2]. It is also the direct precursor to eumelanin pigments [3], and together with a cysteine moiety it forms the pheomelanine pigments [4]. In pigment formation the initial step is the hydroxylation of tyrosine to DOPA by the enzyme tyrosinase (EC 1.14.18.1). Then in a faster reaction also catalysed by this enzyme, DOPA is oxidized to dopaquinone which then can be cyclized to indoles and further to eumelanins. Dopaquinone can also form DOPA thioethers with cysteine, as the basis for biosynthesis of pheomelanins.

For a long time, formation of DOPA in the sympathetic nervous system was considered to be catalysed by tyrosinase. With the isolation of tyrosine hydroxylase (TH, EC 1.14.16.2) [5] it became apparent that sympathetic nervous tissues contain a highly specific enzyme for conversion of tyrosinase into DOPA, and this enzyme is not related to tyrosinase (Fig. 1). The conversion of tyrosine into DOPA by this enzyme is the rate-limiting step in catecholamine biosynthesis [6].

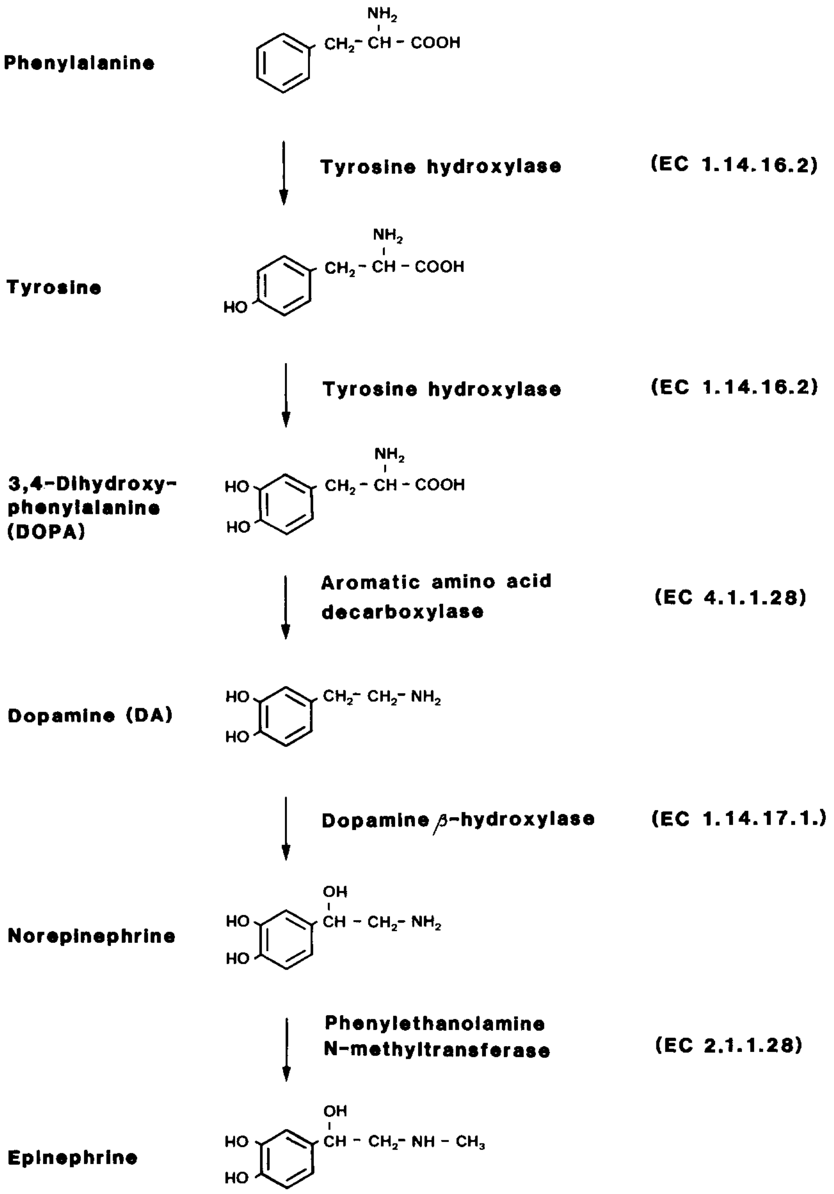


Fig. 1. Formation and metabolism of 3,4-dihydroxyphenylalanine (DOPA).

TH in catecholamine-producing cells uses molecular oxygen and tyrosine as substrate and tetrahydrobiopterin as cofactor to add a hydroxyl group to tyrosine, which results in the formation of DOPA [1,6]. The enzyme can also convert phenylalanine into tyrosine and then into DOPA by successive hydroxylation. In the nervous system, *e.g.* basal ganglia, DOPA is readily decarboxylated to DA by the enzyme DOPA decarboxylase [1] or the more appropriately named aromatic amino acid decarboxylase (AADC, EC 4.1.1.28). This enzyme has pyridoxal phosphate as cofactor. The high content of AADC in the kidney explains why the DOPA concentration in plasma is rather low, and it also explains the mechanism for the contribution of plasma DOPA to the high excretion rate of DA in the urine [7]. Because of its function as a starting material for important neurotransmitters in the nervous system and for build-up of structural elements in pigmented tissue, the determination of DOPA in various tissues and body fluids deserves special attention.

Because of depressed dopaminergic function in Parkinson's disease [2,8,9], L-DOPA is utilized as a therapeutic agent, alone or in combination with dopa decarboxylase inhibitors, in the treatment of this disease [10,11]. The therapeutic effects of L-DOPA are influenced by the dosage [12] and the plasma concentration [13,14], which is also related to side-effects. The demand for monitoring of plasma concentrations has thus also greatly stimulated the development of analytical methods for the determination of DOPA.

2. ANALYTICAL METHODS

When DOPA was introduced as a therapeutic agent in the treatment of Parkinsonism, the demand for the development of appropriate techniques was widened to the determination of the compound in tissue and body fluids. Review articles covering various aspects of analytical methods for catecholamines have been published [15–19]. These included some aspects of methods for the determination of the precursor amino acid DOPA. However, because of limited space in these reviews, only limited attention was paid to this particular catecholic amino acid, although to a large extent the principles for its detection and quantification may be the same as for catecholamines.

2.1. Fluorometric methods

2.1.1. Dihydroxyindole methods

Fluorometric analysis is a sensitive tool for the detection of compounds with fluorogenic properties. This has been used in sensitive methods for DOPA determination. After oxidation, cyclization and isomerization in alkaline media, DOPA forms a dihydroxyindole derivative with fluorescent properties. Iodine [20–24], potassium ferricyanide [25] or sodium periodate [26] oxidize DOPA to a fluorophore in the presence of alkali. With iodine as oxidation agent a pH of 2.9

was originally used [20], later on 5.4 [23], and then a rather broad pH maximum of 5–5.5 was reported [27,28]. During cyclization and rearrangement at high pH, either ascorbate [20,23] or sulphite [23] should be added as an antioxidant. The most effective way of stabilizing the fluorescent derivative is to decrease the pH as soon as possible after rearrangement [23]. With ferricyanide as oxidant, the pH optimum is somewhat higher, 5.5–7.5 [23], but an antioxidant has to be added during rearrangement [25,29]. EDTA must not be present during the oxidation step in either the iodine or ferricyanide methods, or else the specificity of these oxidation reactions will be lost [28]. This effect of EDTA may explain why the iodine oxidation method of Chang [22] is not as specific for DOPA as the Laverty–Taylor method [23]. During clean-up of DOPA from biological samples according to Anton and Sayre [30], EDTA is often used to inhibit oxidation by heavy metals. If, however, EDTA remains in the sample, the amount of ferricyanide has to be increased to obtain effective oxidation in the fluorescent derivatization step [29].

A semiautomated method using iodine oxidation for the measurement of DOPA in plasma was described by Spiegel and Tonchen [31]. After purification, analysis was performed with an AutoAnalyzer equipped with a continuous-flow fluorometer. The limit of detection of the method was 1 μg/l (5 nM).

A similar automated method was later developed by Cottet-Emard and Peyrin [29] using ferricyanide as oxidation agent. They also investigated possible interference from related compounds (Table 1), and found that the reaction was positive only for β-hydroxylated catecholamines [epinephrine (E) and norepinephrine (NE)] but neither for dihydroxyphenylethylamines [dopamine (DA) and epinine] nor for monophenolic amines (tyramine, octopamine, synephrine). No appreciable interference was observed from either the acidic or the neutral catechols studied, or from any of the investigated catecholic compounds. These authors [29] also investigated the urinary excretion of DOPA in different animals and found rather great differences among species (Section 3).

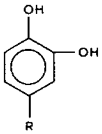
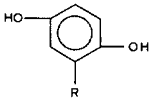
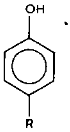
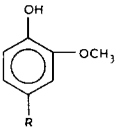
A method for the determination of physiological DOPA concentgrations in urine was described by Türler and Käser [27]. They used combined alumina and ion-exchange clean-up of the urine, and they oxidized DOPA with iodine. With DOPA added to the urine as an internal standard they obtained a mean (± S.D.) recovery of 57.9 ± 10.9%.

With the iodine oxidation method for the determination of DOPA described by McGeer and McGeer [21], 3-O-methyl-DOPA develops only 12% of the fluorescence intensity of DOPA [32]. In patients on DOPA therapy the cerebrospinal fluid (CSF) concentration of 3-O-methyl-DOPA may increase substantially and cause serious errors in DOPA determination. Realizing this, Sharpless and McCann [32] studied critical variables in fluorescence development with potassium ferricyanide for the two compounds, and optimized the methods for DOPA and 3-O-methyl-DOPA alone and in mixtures, and for quantitative analysis in the CSF.

TABLE 1

RELATIVE INTERFERENCE FROM CATECHOLIC AND PHENOLIC COMPOUNDS IN THE FERRICYANIDE METHOD FOR DETERMINATION OF DOPA IN URINE

From Cottet-Emard and Peyrin [29].

Compound	Formula		Relative intensity of fluorescence	Final interference after whole extraction procedure
	Ring	Side-chain R		
Catechols				
DOPA		$CH_2\text{-}\underset{\substack{\vert \\ NH_2}}{CH}\text{-}COOH$	100	100
Dopamine		$CH_2\text{-}CH_2\text{-}NH_2$	0.8	0
Epinine		$CH_2\text{-}CH_2\text{-}NH\text{-}CH_3$	3.6	0
Norepinephrine		$CHOH\text{-}CH_2\text{-}NH_2$	105	0
Epinephrine		$CHOH\text{-}CH_2\text{-}NH\text{-}CH_3$	92	0
DHPG		$CHOH\text{-}CH_2OH$	1	0.5
DOMA		CHOH-COOH	0.65	0.6
DOPAC		$CH_2\text{-}COOH$	0	0
DOBA		COOH	0.25	0.04
DOCi		CH=CH-COOH	1.4	0.24
Pyrocatechol		H	0.15	0.01
2,5-Diphenols				
Homogentisic acid		$CH_2\text{-}COOH$	1	0
Monophenols				
Tyramine		$CH_2\text{-}CH_2\text{-}NH_2$	0.05	0
Tyrosine		$CHOH\text{-}\underset{\substack{\vert \\ NH_2}}{CH}\text{-}COOH$	0.02	0.00002
Octopamine		$CHOH\text{-}CH_2\text{-}NH_2$	0.54	0
p-Sympathol		$CHOH\text{-}CH_2\text{-}NH\text{-}CH_3$	0.19	0
p-Hydroxy-mandelic acid		CHOH-COOH	0.02	0
3-O-Methylated catechols				
MHPG		$CHOH\text{-}CH_2OH$	0.02	0
VMA		CHOH-COOH	0.3	0
HVA		$CH_2\text{-}COOH$	0.2	0

Oxidation agents were investigated and compared by Johnson *et al.* [33]. They found highest fluorescent intensities with KI–I_2 as the oxidant rather than I_2 or $NaIO_4$. They optimized the reagent variables for the oxidation and separation of DOPA from catecholamines, and measured DOPA in rat brain: they found DOPA levels of 28.4 ± 1.4 μg/g wet weight. This was substantially higher than the values obtained by Kehr *et al.* [34].

From the above papers on fluorometric methods it seems evident that hydroxyindole methods have been developed with sufficient sensitivity for the determination of DOPA in blood plasma [28,31], urine [27,29], CSF [32], and brain tissue [28,29,33–35], during both normal and abnormal conditions and during pharmacotherapy. It should be noted that both the sensitivity for detection and the stability of the fluorescent derivative, as well as the specificity of the method for DOPA, differ depending on which oxidation reagent is used. The specificity and recovery are also dependent on the clean-up procedure. The risk of interference from other compounds, whether unexpected or expected, is high with these methods. Thus mainly because of low specificity these methods have been largely replaced by more specific method, such as high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS).

2.1.2. *Ethylenediamine condensation method*

Ethylenediamine condenses with catecholic compounds to yield fluorescent compounds [36]. This reaction was used in a method for simultaneous determination of DOPA, DA and 3,4-dihydroxyphenylacetic acid (DOPAC) in urine after low-voltage paper electrophoresis [37]. Although the method is simple it has not been further validated, nor come into general use.

2.2. *Radiometric techniques*

In the single-isotope radioenzymic methodology for analysis of the catecholamines DA, NE and E, the enzyme catechol-O-methyltransferase (COMT, EC 2.1.1.6) is used to transfer a radioactive methyl group from [methyl-^{3}H]-S-adenosyl-L-methionine ([^{3}H]SAM) to the catecholamine. The radioactive O-methyl catecholamine derivatives are then separated, usually by thin-layer chromatography (TLC), and the derivative from a particular catecholamine is quantified by radioactivity counting (see refs. 15, 16 and 18).

DOPA is also a substrate for COMT. This reaction was used in the straightforward method of Hefti and Lichtensteiger [38], based on the conversion of DOPA into [^{3}H]-O-methyl-DOPA by [^{3}H]SAM in the presence of COMT. The enzyme COMT, however, contains an impurity of DOPA decarboxylase activity, which is more appropriately called aromatic L-amino-acid decarboxylase (AADC, EC 4.1.1.28). An AADC inhibitor must therefore be included to inhibit the further conversion of the radioactive DOPA product into [^{3}H]-O-methyl-DA. Such an inhibitor is also necessary in the determination of DA by a similar procedure [39] in order that falsely high values for DA should not be obtained by conversion of DOPA into DA. In the combined method for DA and DOPA described by Johnson *et al.* [40], DA is determined by including an AADC inhibitor, benzyloxylamine, in the reagent mixture [39], and the sum of DA and DOPA is determined by the addition of AADC to the reagent and omission of the inhibitor. The DOPA level is then obtained by subtraction. A similar procedure described by Brown and Dollery [41] was applied to the determination of DOPA in human plasma and urine.

The radioenzymic technology for DOPA determination was greatly improved by Zürcher and Da Prada [42]. Their assay (Fig. 2) combined O-methylation of

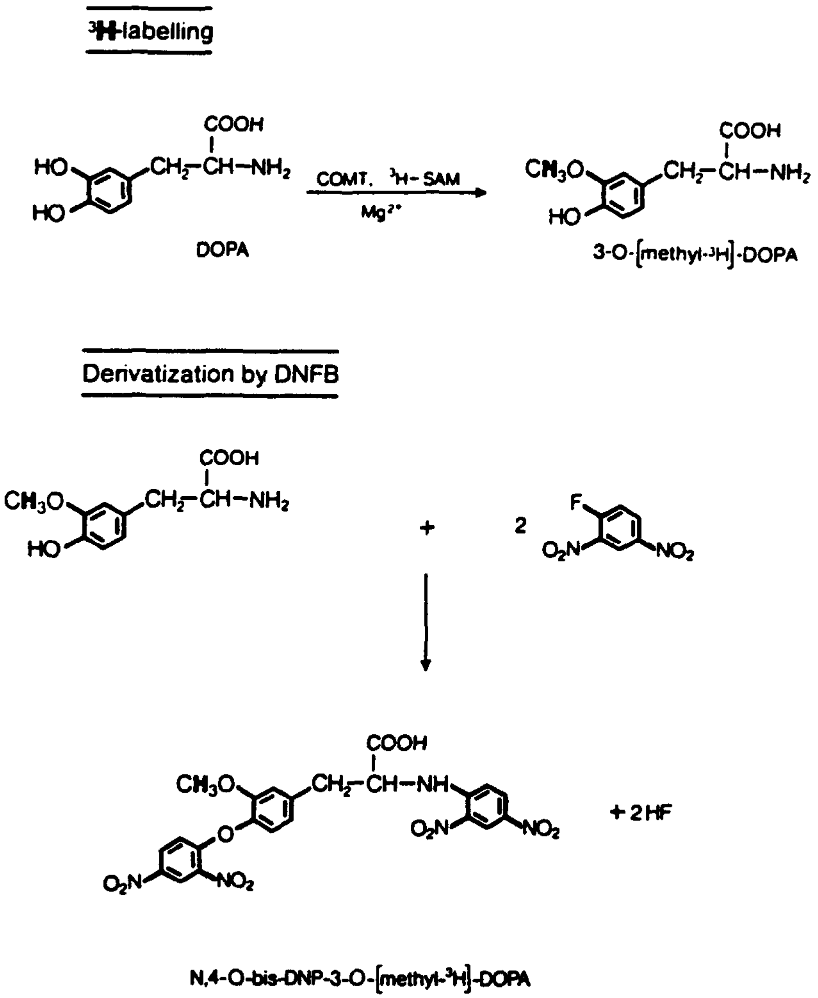


Fig. 2. Tritium labelling of DOPA by enzymic methylation to 3-O-[methyl-^{3}H]DOPA, and subsequent derivatization by 2,4-dinitrofluorobenzene (DNFB). The final product, 3-[4-(2,4-dinitrophenoxy)-3-methoxyphenyl]-N-(2,4-dinitrophenyl)-L-alanine (N,4-O-bis-DNP-3-O-[methyl-^{3}H]DOPA), was identified by structure analysis. From Zürcher and Da Prada [42].

DOPA, by purified COMT with [^{3}H]SAM as the methyl donor, with subsequent derivatization of 3-O-[methyl-^{3}H]DOPA by 2,4-dinitrofluorobenzene. The final product was further purified by solvent extraction and isolation by TLC before radioactive counting. Their method was applicable both to brain tissue and human plasma. The method of Argiolas and Gessa [43] replaced the complicated derivatization and purification by purifying the labelled product on Sephadex G10 and Dowex 50W XY, and thus accomplished a simple procedure for brain tissue.

Thiede and Kehr [44] succeeded in combining various methodological approaches for the single determination of catecholamines, DOPA and the catecholamine metabolites carrying a catechol moiety. Thus their method allows the simultaneous determination of DOPA, DA, NE, E, DOPAC, 3,4-dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylethanol (DOPET) and 3,4-dihydroxyphenylethylene glycol (DOPEG). They reported quantitative analytical recovery, which for plasma DOPA was 98 ± 6.1%.

A quite different approach was reported by Faraj *et al.* [45] for the determination of DOPA (and DA) in urine. They isolated DOPA from urine by ion-exchange chromatography. DOPA was then enzymically decarboxylated by AADC to DA and further converted into 3-O-methyl-DA by COMT. The amount of 3-O-methyl-DA was determined by radioimmunoassay [46]. The mean (±S.D.) analytical recovery of DOPA added to urine was 65 ± 7.5%.

2.3. Chromatographic methods

Chromatographic methods differ from colorimetric, fluorometric and radiometric techniques in one important sense, *viz.* interference from compounds other than the analytes in question can more readily be detected and therefore avoided. This does not mean that the sensitivity of the chromatographic methods is always higher (although it often is), but properly conducted chromatographic methods most often are more specific than non-chromatographic methods.

2.3.1. Ion-exchange techniques (amino acid analyser techniques)

Classically, amino acids are determined in plasma and urine by separation on an ion exchanger, and quantitated post-column by reaction with ninhydrin [47] or orthophthaldialdehyde [48]. DOPA, being an amino acid, was early subjected to such a procedure [49]. Acidified urine and plasma samples were deproteinized with sulphosalicylic acid and chromatographed directly, utilizing a modified program for elution and ninhydrin for detection. The methods had a detection limit of 100 μg/l (0.5 μM) [50], which was sufficient for the estimation of DOPA in plasma and urine in Parkinson disease patients receiving L-DOPA therapy [50]. By connecting a spectrophotofluorometer to the analyser, Hare *et al.* [51] measured the native fluorescence of DOPA and were able to measure its plasma concentration together with some of its metabolites after L-DOPA therapy.

Seki *et al.* [52] utilized a high-performance amino acid analyser and post-column reaction with orthophthaldialdehyde and fluorometric detection to obtain an impresicion of only 2% for the determination of DOPA in urine. The pretreatment of urine was simple, in that they mixed 0.2-ml aliquots of urine with 0.8 ml of 0.1 *M* acetic acid containing dihydroxybenzylamine (DHBA) as internal standard before HPLC. They compared their results with the analysis of urine samples extracted with alumina before analysis, and found virtually identical results.

The use of amperometric detectors for monitoring of DOPA in the effluent from a chromatograph was first described by Kissinger *et al.* [53]. They used a strong cation exchanger for chromatography, and electrochemical detectors and electrodes of various design. Serum was precipitated by perchloric acid and then subjected to alumina extraction. Pooled normal serum gave a peak that indicated a serum concentration of less than 2 μg/l (less than 10 n*M*) in healthy subjects. A following paper [54] described a similar technique for the quantification of DOPA and DA in the same chromatogram. The analytical recovery from serum was 95.1 ± 3.0%, the imprecision was 2.8 and 3.1% (within run and day-to-day, respectively, and the detection limit was 10 μg/l (50 n*M*).

2.3.2. *Reversed-phase high-performance liquid chromatography*

Several problems have to be solved when determining DOPA by HPLC. One has to decide whether DOPA should be determined alone or together with other relevant metabolites. The demand for sensitivity in the method is quite different for the various biological fluids, and whether normal or pharmacological concentrations should be determined.

2.3.2.1. Detection. HPLC with UV detection of DOPA gives satisfactory results in studies on pure pharmacological solutions and its *in vitro* decarboxylation through enzyme reactions [55]. UV detection was also used successfully in the determination of DOPA and its major metabolites in hydrolysed urine of L-DOPA-treated parkinsonian patients [56].

A number of papers describe the monitoring of DOPA in the effluent from liquid chromatography by ED [52–54,57–77]. Most of the earlier studies used amperometric detection with single thin-layer cells from Bioanalytical Systems (West Lafayette, IN, U.S.A.) with different kinds of material, mostly glassy carbon. Recently, as dual electrode from the same company has come into use which enables one to apply different potentials for the detection of interference with the peak of interest (DOPA) or for the simultaneous analysis of other compounds like 3-O-methyl-DOPA oxidizable at higher potential than DOPA [75]. The use of the dual coulometric electrode system made by ESA (Environmental Science Associates, Bedford, MA, U.S.A.) also contributes to improved specificity for DOPA determination [70,72,73]. For further discussion on the differences between amperometric and coulometric detection, the reader is referred to the review on catecholamines by Nyyssönen and Parviainen [19].

Fluorometry has come into use to only a limited extent in liquid chromatography, either for measuring the native fluorescence of DOPA [51,71,78] or for determination of the DOPA derivative with 1,2-diphenylethylenediamine [79]. It was clearly shown by Ishimitsu and Hirose [71] that the detection limit was higher (*ca.* five-fold) with fluorometric than with amperometric detection, although specificity might be better.

2.3.2.2. Separation. Biological fluids contain a large number of electroactive compounds. For effective separation and detection of DOPA it is necessary that DOPA does not co-elute with any other such compound. A few papers have investigated the retention of DOPA on reversed-phase columns, particularly octadecyl columns [58,59,62], and compared the retention with that of relevant compounds, such as catecholamines and other catecholic amino acids. Thus Hansson *et al.* [58], using a mobile phase of 31 m*M* phosphoric acid with Spherisorb ODS and LiChrosorb 6 RP-18 columns, found a decrease in retention when the ionic strength was increased by the addition of 115 m*M* sodium sulphate to the mobile phase. With Nucleosil C_{18} there was a slight increase, and with Partisil no change occurred.

Fig. 3 shows the capacity ratio for DOPA and other catecholic compounds

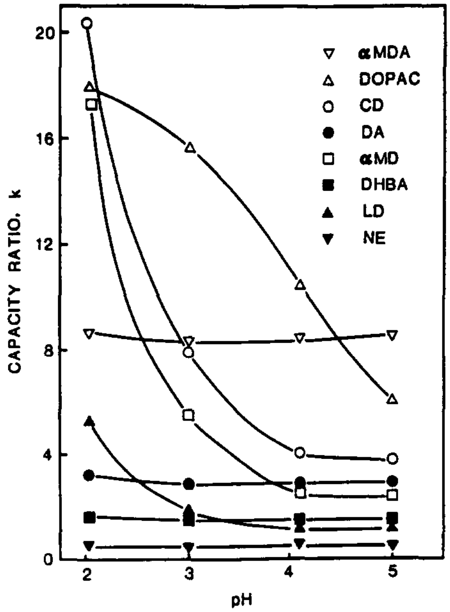


Fig. 3. Effect of mobile phase pH on the capacity ratio, k', of catecholic compounds. Column, μBondapak C_{18} reversed-phase column, 300 mm × 3.9 mm I.D.; mobile phase, 0.1 *M* nitric acid titrated to indicated pH with sodium hydroxide. Compounds: αMDA = α-methyldopamine; DOPAC = 3,4-dihydroxyphenylacetic acid; CD = carbidopa; DA = dopamine; αMD = α-methyldopa; DHBA = 3,4-dihydroxybenzylamine; LD = L-3,4-dihydroxyphenylalanine (DOPA); NE = norepinephrine. From Asmus and Freed [59].

when the pH of the mobile phase was varied between 2 and 5. Note that catecholamines have a constant retention over the pH 2–5 range, whereas the catecholic amino acids [DOPA, α-methyl-DOPA and carbidopa (CD)] show a drastic drop in retention when the mobile phase pH is raised. This is due to the fact that the pK_a of the amino group is well above 5, and the charge of the catecholamines does not change from pH 2 to 5. The pK_a values for DOPA are 2.30 and 9.7 for the –COOH and $-NH_2$ groups, respectively [80]. Thus when the pH of the mobile phase is increased from 2 to 3, the –COOH group becomes deprotonized and DOPA forms the very polar zwitterionic species, which causes the retention to decrease. A similar effect has been described by others [58,61,62], and is also evident for the other carboxylic compounds [α-methyldopamine (αMD), CD, DOPAC] shown in Fig. 3.

Although separation without the addition of an ion-pairing reagent has been described for the quantitation of DOPA in plasma [57,64,67,69] and urine [67], the resolution of DOPA from other catecholic compounds is greatly improved by the addition of an anionic ion-paring reagent [56]. Different kinds of ion-pairing reagents have been used, such as trichloroacetate [59], methanesulphonic acid [58,68], heptanesulphonic acid [56,61,75,76], octanesulphonic acid [61–63,66,70,73,78], octylsulphate [59,64] and dodecylsulphate [72,77]. This greatly enhances the retention of catecholamines (Fig. 4); for the purpose of moderating the retention of the final chromatographic peak, an organic modifier such as methanol or acetonitrile should be added.

An interesting approach for proper separation was the generation of gradient systems, both flow gradients [65] and gradients with organic compounds such as acetonitrile [70] and methanol [71]. The purpose of these systems is to obtain improved separation in the early part of the chromatogram (where DOPA elutes) while ensuring the elution of the final peak within a reasonable time. A drawback may be the variation of the background during chromatography.

2.3.2.3. Sample preparation. As discussed in Section 3, the endogenous concentration of DOPA in biological fluids and tissues is low, and most extracts contain a number of possible interferences, that can give false results with different methods. However, in certain situations, the DOPA concentration may be high, the tissue extract may not contain interferences, or the chromatographic method may be highly specific. Thus, for optimal choice of clean-up procedures such factors have to be taken into consideration.

Five papers can be identified in which the authors achieved protein-free extracts of serum or plasma by adding perchloric or trichloroacetic acid to the serum [69,71,72,74,75]. After separation by centrifugation, the concentration of strong acid in the supernatant was reduced by precipitation with potassium hydroxide or by dilution with water, and after centrifugation 20 μl [69,74,75], 30 μl [72] or 100 μl [71] of the clear extract was injected into the chromatograph, equipped with an electrochemical detector. The absolute recoveries of these procedures were almost quantitative, *i.e.* 98% [69], 100% [71], 94–99% [72], 91% [74]

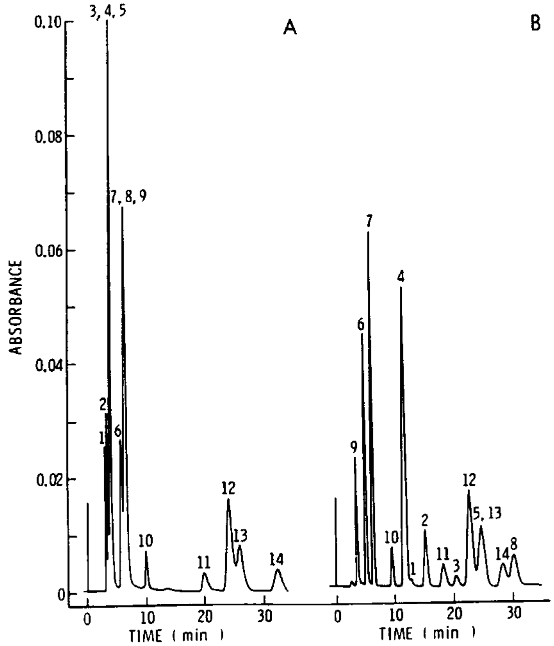


Fig. 4. Chromatogram of a standard mixture of L-DOPA metabolites in the absence (A) and presence (B) of 5 m*M* heptanesulphonic acid. Column, μBondapak C_{18} reversed-phase column, 300 mm × 3.9 mm I.D.; mobile phase, water–methanol–acetic acid. Peaks: 1 = norepinephrine; 2 = epinephrine; 3 = normetanephrine; 4 = DOPA; 5 = DA; 6 = vanillylmandelic acid; 7 = 3-methoxy-4-hydroxyphenylglycol; 8 = 3-methoxy-4-hydroxyphenylalanine; 9 = 3,4-dihydroxyphenylpyruvate; 10 = DOPAC; 11 = vanillyllactic acid; 12 = homovanillic acid; 13 = vanillic acid; 14 = isovanillic acid (3-hydroxy-4-methoxybenzoic acid). From Mitchell and Coscia [56].

and 99% [75], and the precision (coefficient of variation, C.V.) varied between 2.6 and 4.7%. These methods are suitable for the determination of DOPA in serum or plasma from patients on DOPA therapy. The sensitivity of these methods was not sufficient for the determination of endogenous concentration of DOPA, except in one case [71] in which the plasma concentration could be determined in four out of five healthy subjects.

These clean-up procedures are simple, and the analytical results seem to be accurate, but the methods require the use of a precolumn that has to be replaced rather often. Because plasma concentration of DOPA is high after DOPA ingestion, only small amounts of plasma or serum are required for analysis.

A number of papers have described how DOPA from serum or plasma, either directly or after protein precipitation, is adsorbed on alumina at pH *ca.* 8.6. After washing, DOPA is eluted by the addition of an acid, usually perchloric acid, and the eluate is injected into the chromatograph [53,54,57–60,62–66,68,70,73,76] (Fig. 5).

Typical results for detection limit for DOPA in plasma were 1 μg/l (50 n*M*)

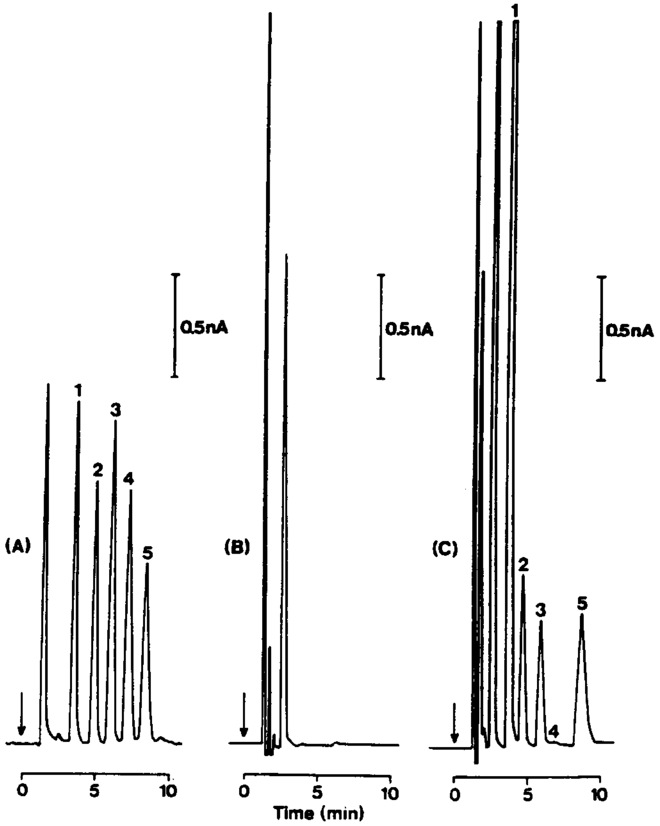


Fig. 5. Chromatograms of (A) catecholamine standards, (B) predose control plasma, and (C) plasma obtained from a volunteer 60 min after oral administration of 100 mg of levodopa with 25 mg of carbidopa and containing DHBA as internal standard. Clean-up of plasma was by adsorption on acid-washed alumina (pH 8.6) and elution with perchloric acid. Column, ODS Spherisorb (particle size 5 μm), 250 mm × 2.5 mm I.D.; mobile phase 100 m*M* NaH_2PO_4, 20 m*M* citric acid, 1.25 m*M* sodium octanesulfonate and 0.15 m*M* sodium EDTA in 8% methanol. The pH was adjusted to 3.2. Peaks: 1 = DOPA; 2 = DHBA; 3 = DOPAC; 4 = DA; 5 = carbidopa. From Nissinen and Taskinen [63].

[63], 25 μg/l (125 n*M*) [66], 0.02 μg/l (0.1 n*M*) [68] and 0.15 μg/l (0.75 n*M*) [76]. Thus, from these data and other data in the papers, it is obvious that endogenous plasma levels can be determined. At these low concentrations there is a high risk that other endogenous compounds may disturb the peak from DOPA. The alumina clean-up procedure makes it possible to concentrate DOPA in the eluate, together with other catecholic compounds. This is one reason why the sensitivity of such a method is high. However, it should be borne in mind that the absolute recoveries were quite variable, *i.e.* 60% [53], 95% [54], 71% [57], 71–76% [58], 57% [60], 61% [63], 68–80% [64], 72% [66], 76% [68], 50% [70], 24% [73] and 58% [76]. To obtain quantitative analytical recoveries the use of a suitable in-

ternal standard is necessary. With such methods the imprecision seems to be generally in the range 2–6%. Similar results were also obtained by clean-up with cation-exchange chromatography: recovery 61% and imprecision 4.6 and 6.2% [79].

Alumina extraction has also been employed for the clean-up of extracts from urine [53,56,58,68] and tissues [60–62,65,68], mainly rat brain tissue. Recoveries are similar to those obtained with plasma, but the urinary chromatograms (Fig. 6) may show more peaks and the chromatograms from tissue fewer.

A few papers may be mentioned that have described other clean-up procedures. Thus, for the clean-up of urine Benedict and Risk [67] used both benzenesulphonic acid-derivatized silica (SCX) and methyl-derivatized silica (C_1), in combination with phenylboronate silica column chromatography. For deproteinized plasma only the latter column was used. As measured by added [^{14}C]DOPA, the recovery in step 1 was 96% and in step 2 it was 80% (urine) and 84% (plasma). No peak was detected at the retention time of DOPA when a urine or plasma sample was preincubated with L-aromatic amino acid decarboxylase. The normal urinary and plasma concentration could well be measured in such purified samples by HPLC and electrochemical detection (ED).

Boomsma *et al.* [77] determined DOPA in plasma either by a direct method similar to that of Baruzzi *et al.* [72] or indirectly by conversion into DA with the AADC enzyme and liquid–liquid extraction of DA before HPLC. The purification procedure was based on extraction of catecholamines as complexes with diphenylboron and tetraoctylammonium bromide into *n*-heptane and back-extraction with 250 μl of 0.08 *M* acetic acid. In 60 plasma samples DOPA values measured with the two methods showed good agreement. Below 25 μM, however, the direct method showed no detectable DOPA or occasionally gave higher values than the conversion method owing to the presence of small unknown substances. The direct method was excellent for the determination of DOPA and 3-O-methyl-DOPA, and the conversion method was suitable for the determination of the endogenous concentrations of DOPA in plasma and cerebrospinal fluid.

Tsuchiya and Hayashi [78] investigated direct extraction into *n*-hexanol via complexation of its catechol moiety with different kinds of boronic acid and its carboxylic group with tetraalkylammonium ion in an alkaline buffer. With diphenylborate and tetrapentylammonium ion, the absolute recovery was 77.2 ± 5.1% and the analytical recovery, with DHBA as internal standard, was 100.8 ± 2.3%. Although not shown in the paper, the authors state that quantitative results obtained with their HPLC method were in good agreement with those obtained by their GC–MS method.

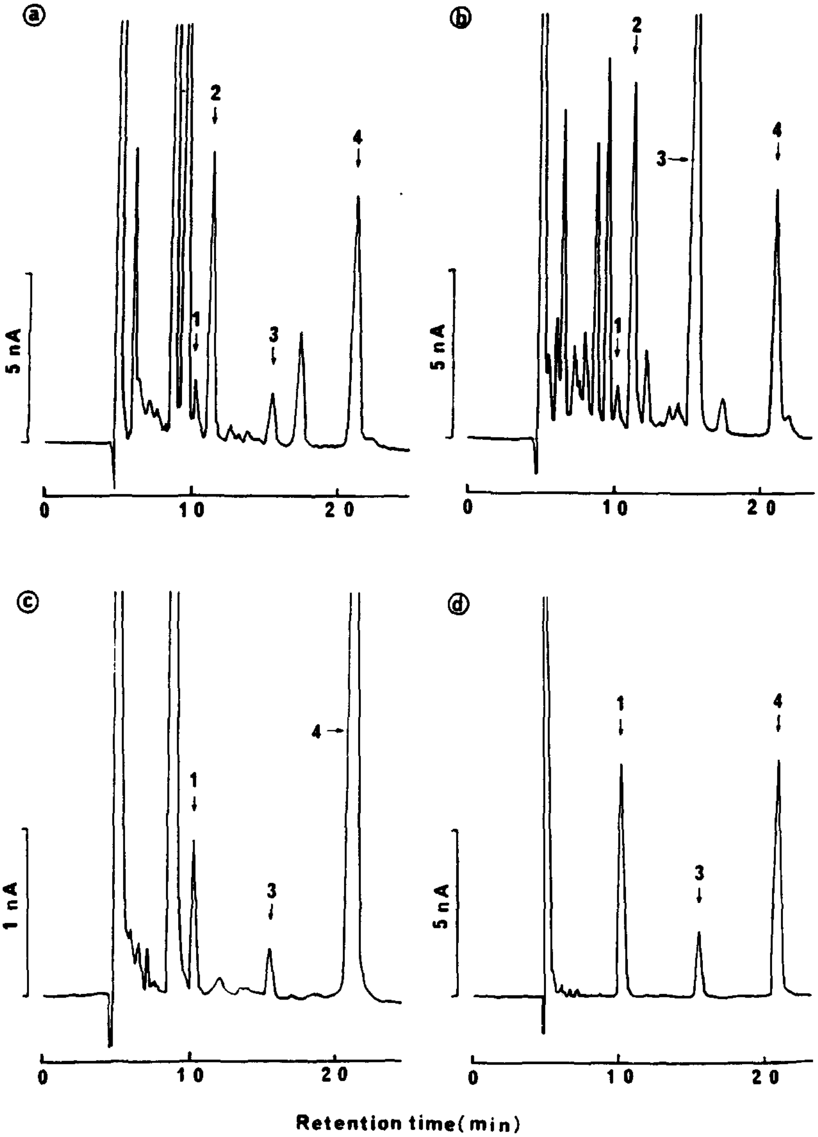


Fig. 6. Chromatograms of (a) a urine sample from a normal subject, (b) a urine sample from a melanoma patient, (c) a plasma sample from a normal subject, and (d) an extract of a B16 mouse melanoma. Column, Yanaco ODS-A (7 μm particle size); mobile phase, 10 g/l phosphoric acid and 7 g/l methanesulphonic acid, pH adjusted to 2.35 with 5 *M* sodium hydroxide and Na_2EDTA added to a final concentration of 0.1 m*M*; flow-rate, 0.7 ml/min; temperature, 40°C; detector, +750 mV *versus* Ag/AgCl, 4 and 32 nA at full scale. Peaks: 1 = DOPA; 2 = dopamine; 3 = 5-S-cysteinyl-DOPA; 4 = α-methyl-DOPA. From Ito *et al.* [68].

2.3.3. *Gas chromatography*

2.3.3.1. Flame ionization detection. Contamination of drugs with impurities has always to be taken into consideration. This is of particular importance for L-DOPA therapy, because large daily doses (6–8 g) may be given; an impurity level of 1% would result in the intake of 60–80 mg per day of an extraneous, maybe closely related, metabolically active amino acid. GC with flame ionization detection was considered suitable for this purpose because since it may be specific to the determination of L-DOPA and, at the same time, non-selective in the sense that each contaminant in the sample could be identified and quantified. Such a method was developed by Chang *et al.* [81], who used N,O-bis(trimethylsilyl)-acetamide as the silylating reagent. In different commercial levodopa capsules and tablets they found up to 1.27% of contaminants, including 3-aminotyrosine, 3-methoxytyrosine, 3,4,6-trihydroxyphenylalanine, tyrosine and phenylalanine.

2.3.3.2. Electron-capture detection (ECD). In their studies on L-DOPA metabolism Imai *et al.* [82] first employed a GC–ECD procedure, in which the amino acid was converted into a trifluoroacetyl oxazolone. In a following paper [83] they were able to increase the sensitivity of the method by first forming a DOPA *n*-butyl ester with *n*-butanol saturated with HCl gas. Then the trifluoroacetyl derivative was obtained with trifluoroacetic anhydride, and the derivative was separated and detected by GC–ECD. The presence of DOPA in urine was demonstrated by comparing the mass spectrum from GC of the TFA derivative of authentic DOPA *n*-butyl ester with that obtained from urine [83].

Two years later, Watson [84] applied GC to the detection and quantitation of impurities in levodopa raw material and commercial dosage forms. Of the silylation reagents tested, N,O-bis(trimethylsilyl)acetamide in acetonitrile was found to be the most suitable for DOPA and the proposed possible impurities (3,4-dimethoxyphenylalanine, L-tyrosine, 3-hydroxy-4-methoxyphenylalanine, 3-methoxy-4-hydroxyphenylalanine and 6-hydroxydopa). Only 3-methoxy-4-hydroxyphenylalanine was detected as a contaminant in five commercial products examined.

With a modification of the method of Imai *et al.* [83], Mizuno [85] developed a GC–ECD method for the determination of DOPA and dopamine in serum. They used α-methyl-DOPA as internal standard. After alumina extraction of DOPA from 1 ml of serum, derivatization was performed first with HCl-saturated butanol and then with trifluoroacetic anhydride. The recovery of DOPA added to plasma was 84%, and the analytical range was suitable for the detection of L-DOPA in plasma after intake either alone or together with DOPA decarboxylase inhibitors.

2.3.3.3. Mass spectrometric detection. The method for the determination of DOPA and 3-O-methyl-DOPA in plasma and CSF described by De Jong *et al.* [86] takes advantage of selected-ion monitoring during electron-capture negative-ion (ECNI) chemical ionization conditions. The derivative used was the N,O-acetylpentafluorobenzyl ester of DOPA, and the limit of detection at a signal-tonoise ratio of more than 5 was *ca.* 0.1-0.2 pg injected, using selected-ion monitoring of the carboxylate anion. With the extraction and derivatization techniques used for plasma and CSF they obtained an analytical recovery for DOPA of 100.2 ± 3.7% at the level of 100 n*M*. The imprecision (C.V.) of the method was 5%, and the limit of quantitation in plasma and CSF was at the sub-nanomolar level.

A slightly modified method was described by Hayashi and Tsuchiya [87]. They used $^{2}H_{6}$-labelled L-DOPA as the internal standard, and applied the method to determine plasma levels of deuterated and non-deuterated L-DOPA after oral administration of $^{2}H_{3}$-labelled L-DOPA to a healthy subject.

2.4. Concluding remarks on analytical methods

It is clear that over the years different kinds of analytical methods have been successfully used. For the time being older methods, such as fluorometric methods, have been largely abandoned, mainly because of their uncertain specificity. To some extent this is also true of radiometric techniques. However, it should be pointed out that important research has been done with these methods, and most of the results obtained still hold true. It is a pity that comparative studies have not been done between these methods and HPLC or GC–MS. Normal plasma reference levels found with different kinds of method are in reasonable agreement (see Section 3).

Liquid chromatographic methods have expanded greatly during the past twenty years. They are ideal for the measurement of plasma DOPA after intake of L-DOPA, for example, because of quantitative absolute and analytical recoveries. They are also straightforward and show good reproducibilities. As far as the determination of endogenous levels in plasma, CSF, urine and tissues is concerned, these methods are susceptible to interference, and great care must be taken to avoid co-chromatography with other components in the body fluids. With these methods, absolute recoveries are variable, and internal standard techniques have to be used to compensate for this variability.

GC–MS methods for determination of DOPA are scanty. These methods are laborious and require expensive equipment and great skill.

It can be concluded that methodological research and improvements still have to be carried out to meet the standards required for the determination of DOPA.

3. BIOLOGICAL APPLICATIONS

3.1. Reference (normal) values in humans

The normal concentration of DOPA has to be established in order that proper comparison is possible between healthy and diseased subjects. For individual subjects the comparison is best done with normal reference ranges, most often determined as the mean ± 2 S.D. for healthy subjects. When diseased groups are to be compared, the mean and the S.D. (or S.E.M.) are calculated for significance testing, or else non-parametric tests are employed.

3.1.1. Human plasma/serum

Values of human plasma/serum concentration of DOPA determined by different methods are listed in Table 2. (Data have been recalculated to SI units if given otherwise in the original papers.) Fluorometric, radioenzymic and liquid chromatographic methods have been used in efforts to elucidate differences between the sexes, but no such difference has been detected for plasma/serum DOPA. Furthermore, Boomsma *et al.* [77], who used HPLC, did not find any age difference. It seems, therefore, that a rough comparison of reference values obtained with

TABLE 2

PLASMA/SERUM CONCENTRATIONS OF DOPA IN HEALTHY HUMAN SUBJECTS

Values are given in nM.

Method	Men		Women		Both sexes		Subject (H = healthy)	Ref.
	n	Mean ± S.D. (range)	*n*	Mean ± S.D. (range)	*n*	Mean ± S.D. (range)		
Fluorometric	9	14.6 (9.9–18.4)	3	17.4 (14.4–9.0)	12	15.2 ± 3.5 (9.9–19.0)	H Other	79
					7	9.8 ± 4.6		
Radioenzymic	18	6.7 ± 0.4 (4.7–10.1)	24	7.7 ± 0.3 (47.–12.1)	42	7.3 ± 0.1 (4.7 ± 12.1)	H	49
	10	9.2 ± 3.1 (4.6–15.9)					H, supine	88
	10	8.6 ± 3.0 (5.9–15.9)					H, standing	
HPLC					20	31.9 (10.1–50.7)	H	57
	6	12.3 ± 1.9	5	12.7 ± 2.9			H	64
					22	5.8 (3.9–9.3)	H	65
					7	10.8 ± 1.6	H	67
					31	4.3 ± 1.9	H	68
					9	10.5 (5.6–15.2)	H	70
	9	14.6 (9.9–18.4)	3	17.4 (14.4–19.0)	12	15.3 ± 3.5	H	79
					36	9.5 ± 2.3	H	76
					39	10.6 ± 2.8	H	77
					60	12.6 ± 4.4	Children	77
GC					11	9.0 ± 2.0		86

different methods could be done by using results for adults irrespective of age and sex.

Considering men and women together, mean results were in the range 4.3–31.9 n*M*, as determined with HPLC methods. When the most extreme mean values are excluded, we obtain a range for mean values of 5.8–15.3 n*M*. Mean results from fluorometric, radioenzymic and GC methods are in the middle of this range, which leads to the conclusion that no methodological differences can be traced from the determinations of plasma/serum DOPA.

3.1.2. Human urine

Normal values for human urine are scanty. Türler and Käser [27] used a fluorometric method to determine the urinary excretion of DOPA in eight normal subjects, and obtained the value 111 nmol/24 h (range 30–167 nmol/24 h). With radioenzymic methods, the measured excretion differed considerably, from 6.8 ± 1.5 nmol/24 h ($n = 10$) [46] to 152 nmol/24 h ($n = 4$) [89], and 239 ± 56 nmol/24 h [42]. With HPLC the urinary concentration was determined for men and women to 280 ± 6.2 n*M* ($n = 7$) [79] and 302 ± 49 n*M* ($n = 7$) [67]. With these scanty results we conclude the normal values remain to be estimated conclusively.

3.1.3. Human cerebrospinal fluid

With GC, a mean (S.D.) CSF DOPA concentration of 3.5 ± 0.9 n*M* was found by de Jong *et al.* [86] in nine subjects.

3.1.4. Other human biological fluids

Results for normal saliva, amniotic fluid and milk were given by Shum *et al.* [64]. The analyses were made on occasional patients, and imply a similar or lower DOPA concentration in breast milk (4.6 n*M*) and in amniotic fluid (6.4 and 10.9 n*M*) than in plasma (12.7 ± 2.9 n*M*, $n = 5$). The saliva, however, showed a much lower concentration (0.7 n*M*) than the other biological fluids.

3.2. Clinicopathological values

For the evaluation of DOPA concentrations in body fluids in different clinical situations, it is essential to compare with results obtained from healthy subjects. Thus Boomsma *et al.* [77] determined the plasma concentration by HPLC in healthy subjects, in patients with hypertension and in untreated parkinsonian patients (Table 3). No differences were observed within these categories.

Urinary data from untreated parkinsonian patients are scanty. In two such patients, Mitchel and Coscia [56] used HPLC to obtain a urinary excretion rate of 2.0 μmol/24 h, compared with the normal rate of 7.7. ± 3.1 μmol/24 h in five healthy subjects. Türler and Käser [27] used a fluorometric method to investigate the urinary DOPA excretion in tumours of neuroectodermal origin, and found increased excretion of DOPA in both neuroblastoma and malignant melanoma

TABLE 3

COMPARISON OF PLASMA CONCENTRATIONS OF DOPA IN HYPERTENSIVE PATIENTS AND PARKINSONIAN PATIENTS WITH HEALTHY SUBJECTS [77]

Subject	*n*	Plasma DOPA (n*M*)
Healthy men and women	39	10.6 ± 2.8
Healthy children, 0–15 years	60	12.6 ± 4.4
Hypertensive adults	40	11.1 ± 3.1
Untreated Parkinsonian patients	30	11.4 ± 3.7

(Table 4). Similar findings were obtained by Faraj *et al.* [45], using a radioenzymic method. Thus the mean (±S.D.) urinary DOPA excretion rate of eight patients with stage III melanoma was 642 ± 141 nmol/24 h, compared with the normal rate of 6.8 ± 1.5 nmol/24 h in ten healthy subjects. From these studies it seems

TABLE 4

URINARY EXCRETION OF DOPA IN NORMAL INDIVIDUALS AND IN PATIENTS WITH NEURO-ECTODERMAL TUMOURS

From Türler and Käser [27].

	Number	Age (years)	Urinary DOPA			
			nmol/24 h		µmol/mol creatinine	
Normal						
Adults	8	16–72	Mean:	111	Mean:	10.9
			Range:	30–167	Range:	4.7–17
Children	29	1–9	Mean:	120	Mean:	55.3
			Range:	56–213	Range:	20–110
Phaeochromocytoma	4	40–66	Mean:	101	Mean:	10.3
			Range:	35–137	Range:	2.9–16
Ganglioneuroma	2	2–8	Mean:	63	Mean:	17.2
			Range:	35–91	Range:	16–19
Neuroblastoma	12	1–9	Mean:	872	Mean:	335
			Range:	248–2535	Range:	48–800
Malignant melanoma						
Melanotic (distant metastases)	19	39–65	Mean:	2322	Mean:	298
			Range:	223–8620	Range:	11–1430
Melanotic (primary and/or local metastases)	14	45–78	Mean:	299	Mean:	34.3
			Range:	177–421	Range:	15–745
Partially melanotic	4	58–65	Mean:	253	Mean:	33.2
			Range:	177–294	Range:	26–42
Amelanotic	2	44–57	Mean:	157	Mean:	17.2
			Range:	147–162	Range:	17–18

that DOPA in urine often is increased in patients with malignant melanoma.

Hansson *et al.* [57], using HPLC for plasma determination of DOPA, examined seven patients with melanoma and found the DOPA concentration to vary between 13.2 and 28.9 in six of them, but in one case the plasma DOPA concentration was 243 n*M*. These data should be compared with the normal reference range of 10.1–50.7 n*M*, obtained from twenty healthy individuals.

It is of interest to describe the scanty data obtained from CSF. Since normal values are very difficult to obtain from healthy subjects, data from different categories of patients have to be evaluated. Thus Boomsma *et al.* [77] found mean (±S.D.) concentrations of 4.5 ± 2.4 n*M* in ten patients with untreated depression, and in twelve unspecified non-parkinson neurology patients the mean value was 5.2 ± 1.3 n*M*. These HPLC data were comparable with the findings of 2.5 ± 0.4 n*M* in ten non-psychiatric patients, obtained by Zürcher and Da Prada [42] with a radioenzymic technique.

3.3. Results during L*-DOPA therapy*

With the introduction of L-DOPA treatment during the 1960s a new era in the management of Parkinson's disease began [90]. Initial problems with side-effects due to peripherally formed dopamine could be overcome by addition of DOPA decarboxylase inhibitors [10]. The L-DOPA treatment provides symptomatic relief to most patients at the initial stages of disease, although unpredictable symptom fluctuations in the effect of L-DOPA are seen in advanced Parkinson's disease [91]. The finding that a constant plasma concentration of L-DOPA, maintained by intravenous infusion, stabilizes the symptoms [92] have focused interest on the pharmacokinetics of L-DOPA [93].

Besides intravenous infusion, other modes of administration of L-DOPA have been tested and correlated to clinical effects and plasma concentrations. Thus, continuous intraduodenal administration produces steady plasma concentrations of DOPA with a corresponding reduction in motor fluctuations, whereas other enteral routes produce more variation in the DOPA concentration and the clinical response [94].

Furthermore, continuous intake of L-DOPA solution orally can produce stable DOPA serum levels and approximate the clinical improvement seen with intravenous infusion [95]. As motor fluctuations can still be seen in patients receiving continuous parenteral infusions of L-DOPA, it has been postulated that cerebral mechanisms related to the interaction of DOPA with neural elements are of importance [96]. Thus, studies on the concentrations of DOPA and its metabolites in plasma and CSF have suggested that factors within the brain are of importance in maintaining an optimal response [97].

Pharmacokinetic studies have revealed that DOPA disappears rapidly from the plasma in a biphasic manner, with a half-life of 5–10 min during the distribution phase [98]. The elimination half-life is estimated to be 40–80 min without decarboxylase inhibitor, and *ca.* 130 min with the decarboxylase inhibitor carbidopa [98]. Thus, the extremely rapid distribution and the short elimination phase are responsible for the rapid fluctuations in plasma DOPA. In further studies much interest has been focused on the relationship between plasma concentrations of DOPA and the clinical response [99–101].

4. CONCLUSION

A number of methods have been described for the determination of DOPA in biological fluids and tissues. Earlier methods, such as fluorometric and radioenzymic methods, greatly advanced our knowledge of DOPA, particularly its presence in the brain and its metabolism in neuronal and peripheral tissue.

With the advent of HPLC techniques, adequate methods for the determination of DOPA in plasma were developed. These methods are the basis for much of our present knowledge of, for example, the pharmacokinetics of DOPA.

In comparison with the methodological literature on catecholamines in plasma and urine, the number of publications on DOPA is limited. This is also the case regarding HPLC methods, and GC–MS methods are really few. Comparisons of results obtained with different methods involving HPLC or GC–MS are lacking and, despite rapid development of HPLC techniques during the past few years, reliable knowledge about the normal urinary excretion is also lacking.

The well known L-DOPA therapy is still the therapy of first choice in patients with Parkinson's disease. This has greatly influenced the development of analytical methods for DOPA, and we believe that further development of alternative drug medications will further stimulate the research on DOPA metabolism and its concentration in biological fluids, particularly plasma and CSF.

5. ACKNOWLEDGEMENT

Preparation of this review was supported by the Swedish Cancer Society (Project 2357-B91-05XBC).

REFERENCES

1 N. Weiner and B. B. Molinoff, in G. Siegel, B. Afranoff, R. W. Albers and P. Molinoff (Editors), *Basic Neurochemistry, Molecular, Cellular and Medical Aspects*, Raven Press, New York, 1989, 14th ed., p. 233.

2 O. Hornykiewicz and S. J. Kish, *Adv. Neurol.*, 45 (1986) 19.

3 H. S. Mason, in W. Montagna and F. Hu (Editors), *Advances in Biology of the Skin*, Vol. VIII, Pergamon, Oxford, 1966, p. 293.

4 R. Carstam, C. Hansson, C. Lindbladh, H. Rorsman and E. Rosengren, *Acta Derm. Venerol.*, 67 (1987) 100.

5 T. Nagatsu, M. Levitt and S. Udenfriend, *J. Biol. Chem.*, 239 (1964) 2910.

6 S. Udenfriend, *Pharmacol. Rev.*, 18 (1966) 43.

7 T. L. Sourkes and B. D. Drujan, *Can. J. Biochem. Physiol.*, 35 (1957) 711.

8 G. W. Pearce, *Recent Adv. Neuropathol.*, 1 (1979) 299.

9 B. Scatton, L. Roquier, F. Javoy-Agid and Y. Agid, *Neurology (N.Y.)*, 32 (1982) 1039.

10 G. C. Cotzias, P. P. Papavasiliou and R. Gellene, *N. Engl. J. Med.*, 280 (1969) 337.

11 M.-H. Marion, F. Stocchi, N. P. Quin, P. Jenner and C. D. Marsden, *Adv. Neurol.*, 45 (1986) 493.

12 E. Melamed, in W. C. Koller (Editor), *Handbook of Parkinson's Disease*, Marcel Dekker, New York, Basel, 1989, p. 355.

13 R. J. Hardie, J. Lees and G. M. Stern, *Adv. Neurol.*, 45 (1986) 487.

14 C. Albani, R. Asper, S. S. Hacisalihzade and G. Baumgartner, *Adv. Neurol.*, 45 (1986) 497.

15 P. P. M. Holly and H. L. J. Makin, *Anal. Biochem.*, 128 (1983) 257.

16 I. J. Kopin, *Pharmacol. Rev.*, 37 (1985) 333.

17 A. M. Krstulovic (Editor), *Quantitative Analysis of Catecholamines and Related Compounds*, Ellis Horwood, Chichester, 1986.

18 B. Kågedal and D. S. Goldstein, *J. Chromatogr.*, 429 (1988) 177.

19 K. Nyyssönen and M. T. Parviainen, *Crit Rev. Clin. Lab. Sci.*, 27 (1989) 211.

20 T. L. Sourkes and G. F. Murphy, *Methods Med. Res.*, 9 (1961) 147.

21 E. G. McGeer and P. L. McGeer, *Can. J. Biochem. Physiol.*, 40 (1962) 1141.

22 C. G. Chang, *Int. J. Neuropharmacol.*, 3 (1964) 643.

23 R. Laverty and K. M. Taylor, *Anal. Biochem.*, 22 (1968) 269.

24 G. M. Tyce, M. D. Muenter and C. A. Owen, Jr., *Mayo Clin. Proc.*, 45 (1970) 438.

25 H. Takahashi and T. B. Fitzpatrick, *J. Invest. Dermatol.*, 42 (1964) 161.

26 A. H. Anton and D. F. Sayre, *J. Pharmacol.*, 145 (1964) 326.

27 K. Türler and H. Käser, *Clin. Chim. Acta*, 32 (1971) 41.

28 S. Fahn, A. L. N. Prasad and R. Delesie, *Anal. Biochem.*, 46 (1972) 557.

29 J. M. Cottet-Emard and L. Peyrin, *J. Neural Transm.*, 41 (1977) 145.

30 A. H. Anton and D. F. Sayre, *J. Pharmacol. Exp. Ther.*, 138 (1962) 360.

31 H. E. Spiegel and A. E. Tonchen, *Clin. Chem.*, 16 (1970) 763.

32 N. S. Sharpless and D. S. McCann, *Clin. Chim. Acta*, 31 (1971) 155.

33 J. C. Johnson, G. J. Gold and D. H. Clouet, *Anal. Biochem.*, 54 (1973) 129.

34 W. Kehr, A. Carlsson and M. Lindquist, *Naunyn Schmiedeberg's Arch. Pharmacol.*, 274 (1972) 273.

35 F. Geissbuehler, *Clin. Chim. Acta*, 45 (1973) 423.

36 S. Natelson, J. K. Lugovoy and J. B. Pincus, *Arch. Biochem. Biophys.*, 23 (1949) 157.

37 F. Eichhorn, A. Rutenberg and E. Kott, *Clin. Chem.*, 17 (1971) 296.

38 F. Hefti and W. Lichtensteiger, *J. Neurochem.*, 27 (1976) 647.

39 J. D. Peuler and G. A. Johnson, *Life Sci.*, 21 (1977) 625.

40 G. A. Johnson, J. M. Gren and R. Kupiecki, *Clin. Chem.*, 24 (1978) 1927.

41 M. J. Brown and C. T. Dollery, *Br. J. Clin. Pharmacol.*, 1 (1981) 79.

42 G. Zürcher and M. Da Prada, *J. Neurochem.*, 33 (1979) 631.

43 A. Argiolas and G. L. Gessa, *J. Neurochem.*, 36 (1981) 290.

44 H.-M. Thiede and W. Kehr, *Naunyn Schmiedeberg's Arch. Pharmacol.*, 318 (1981) 19.

45 B. A. Faraj, D. H. Lawson, D. W. Nixon, D. R. Murray, V. M. Camp, F. M. Ali, M. Black, W. Stacciarini and Y. Tarcan, *Clin. Chem.* 27 (1981) 108.

46 B. A. Faraj, V. M. Camp, A. W. Pruitt, J. W. Isaacs and F. M. Ali, *J. Nucl. Med.*, 18 (1977) 1027.

47 W. H. Stein and S. Moore, *J. Biol. Chem.*, 211 (1954) 915.

48 M. Roth and Y. Hanpai, *J. Chromatogr.*, 83 (1973) 353.

49 T. A. Hare and W. H. Vogel, *Biochem. Med.*, 4 (1970) 277.

50 T. A. Hare, R. A. Beasley, R. A. Chambers, D. H. Boehme and W. H. Vogel, *Clin. Chim. Acta*, 45 (1973) 273.

51 T. A. Hare, B. L. Beasley, S. M. deSimone and W. H. Vogel, *Biochem. Med.*, 11 (1974) 305.

52 J. Seki, Y. Arakawa, K. Imai, Z. Tamura, S. Yoshiue, Y. Mizuno, K. Yamada, M. Tatue and H. Narabayashi, *Chem. Pharm. Bull.*, 29 (1981) 789.

53 P. T. Kissinger, L. J. Felice, R. M. Riggin, L. A. Pachla and D. C. Wenke, *Clin. Chem.*, 20 (1974) 992.

54 R. M. Riggin, R. Alcorn and P. T. Kissinger, *Clin. Chem.*, 22 (1976) 782.
55 V. D. Gupta and A. Gupta, *J. Pharm. Sci.*, 69 (1980) 1145.
56 J. Mitchell and C. J. Coscia, *J. Chromatogr.*, 145 (1978) 295.
57 C. Hansson, L.-E. Edholm, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren, *Clin. Chim. Acta*, 88 (1978) 419.
58 C. Hansson, G. Agrup, J. Rorsman, A.-M. Rosengren and E. Rosengren, *J. Chromatogr.*, 162 (1979) 7.
59 P. A. Asmus and C. R. Freed, *J. Chromatogr.*, 169 (1979) 303.
60 C. R. Freed and P. A. Asmus, *J. Neurochem.*, 32 (1979) 163.
61 J. Wagner, M. Palfreyman and M. Zraika, *J. Chromatogr.*, 164 (1979) 41.
62 R. L. Michaud, M. J. Bannon and R. H. Roth, *J. Chromatogr.*, 225 (1981) 335.
63 E. Nissinen and J. Taskinen, *J. Chromatogr.*, 231 (1982) 459.
64 A. Shum, G. R. Van Loon and M. J. Sole, *Life Sci.*, 31 (1982) 1541.
65 S. L. Rossetti, G. Mercuro and C. A. Rivano, *Life Sci.*, 33 (1983) 2387.
66 R. Causon and M. J. Brown, *J. Chromatogr.*, 277 (1983) 115.
67 C. R. Benedict and M. Risk, *J. Chromatogr.*, 317 (1984) 27.
68 S. Ito, K. Toshiaki, K. Maruta, K. Fujita and T. Kurahashi, *J. Chromatogr.*, 311 (1984) 154.
69 M. Beers, M. Stern, H. Hurtig, G. Melvin and A. Scarpa, *J. Chromatogr.*, 336 (1984) 380.
70 D. S. Goldstein, R. Stull, R. Zimlichman, P. D. Levinson, H. Smith and H. R. Keiser, *Clin. Chem.*, 30 (1984) 815.
71 T. Ishimitsu and S. Hirose, *J. Chromatogr.*, 337 (1985) 239.
72 A. Baruzzi, M. Contin, F. Albani and R. Riva, *J. Chromatogr.*, 375 (1986) 165.
73 G. Eisenhofer, D. S. Goldstein, R. Stull, H. R. Keiser, T. Sunderland, D. L. Murphy and I. J. Kopin, *Clin. Chem.*, 32 (1986) 2030.
74 M. A. Mena, V. Muradas, E. Bazan, J. Reiriz and J. G. deYebenes, *Adv. Neurol.*, 45 (1986) 481.
75 J. M. Cedarbaum,. R. Williamson and H. Kutt, *J. Chromatogr.*, 415 (1987) 393.
76 A. Premel-Cabic and P. Allain, *J. Chromatogr.*, 434 (1988) 187.
77 F. Boomsma, F. A. J. van der Hoorn, A. J. Man in 't Veld and M. A. D. H. Schalekamp, *Clin. Chim. Acta*, 178 (1988) 59.
78 H. Tsuchiya and T. Hayashi, *J. Chromatogr.*, 491 (1989) 291.
79 M. Lee, H. Nohta, K. Ohtsubo, B. Yoo and Y. Ohkura, *Chem. Pharm. Bull.*, 35 (1987) 235.
80 J. E. Gorton and R. F. Jameson, *J. Chem. Soc. (A)*, 11 (1968) 2615.
81 B. L. Chang, B. F. Grabowski and W. G. Haney, Jr., *J. Pharm. Sci.*, 62 (1973) 1337.
82 K. Imai, M. Sugiura, H. Kubo, Z. Tamura, K. Ohya, N. Tsunakawa, K. Hirayama and H. Narabayashi, *Chem. Pharm. Bull.*, 20 (1972) 759.
83 K. Imai, N. Arizumi, M.-T. Wang, S. Yoshiue and Z. Tamura, *Chem. Pharm. Bull.*, 20 (1972) 2436.
84 J. R. Watson, *J. Pharm. Sci.*, 63 (1974) 96.
85 Y. Mizuno, *Clin. Chim. Acta*, 74 (1977) 11.
86 A. P. J. M. de Jong, R. M. Kok, C. A. Cramers, S. K. Wadman and E. Haan, *Clin. Chim. Acta*, 171 (1988) 49.
87 T. Hayashi and H. Tsuchiya, *Proc. Jpn. Soc. Med. Mass. Spectrom.*, 13 (1988) 229.
88 G. A. Johnson, C. A. Baker and R. T. Smith, *Life Sci.*, 26 (1980) 1591.
89 S. Demassieux, L. Corneille, S. Lachante and S. Carrière, *Clin. Chim. Acta*, 115 (1981) 377.
90 N. P. Quinn, in W. C. Koller (Editor), *Handbook of Parkinson's Disease*, Marcel Dekker, New York, Basel, 1987, p. 317.
91 C. D. Marsden and J. D. Parkes, *Lancet*, i (1976) 292.
92 M. H. Marion, F. Stocchi, N. P. Quin, P. Jenner and C. D. Marsden, *Clin. Neuropharmacol.*, 9 (1986) 165.
93 J. G. Nutt, *Ann. Neurol.*, 22 (1987) 535.
94 R. Kurlan, J. G. Nutt, W. R. Woodward, K. Rothfield, D. Lichter, C. Miller, J. H. Carter and I. Shoulson, *Ann. Neurol.*, 23 (1988) 589.

95 J. P. Bennett, M. Turk and E. Landow, *Clin. Neuropharmacol.,* 12 (1989) 285.

96 M. M. Mouradian and T. N. Chase, *Clin. Neuropharmacol.,* 11 (1988) 378.

97 R. Durso, G. Szabo, H. Davoudi and R. G. Feldman, *Clin. Neuropharmacol.,* 12 (1989) 384.

98 J. G. Nutt, in W. C. Koller (Editor), *Handbook of Parkinson's Disease,* Marcel Dekker, New York, Basel, 1987, p. 339.

99 M. N. Rossor, J. Watkins, M. J. Brown, J. L. Reid and C. T. Dollery, *J. Neurol. Sci.,* 46 (1980) 385.

100 I. Shoulson, G. A. Glaubiger and T. N. Chase, *Neurology,* 25 (1975) 1144.

101 J. G. Nutt and W. R. Woodward, *Neurology,* 36 (1986) 739.