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SHORT COMMUNICATIONS

Isolation of metabolites of L-dopa—a possible source of error

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A COMBINATION of ion-exchange and adsorption chromatography frequently is used for the isolation of 3,4-dihydroxyphenylalanine (dopa) and its metabolites.[1–6] The method involves adjusting a protein-free extract to pH 2 and passing it through a strong cation-exchange resin (Dowex-50); amino acids and amines are retained by the resin. The amino acids are eluted with a neutral buffer, and then the amines are eluted with strong acid. Deaminated acidic and neutral compounds and glucuronides and sulfates of other metabolites are considered not to be retained by the resin and to be present in the effluent. In all fractions, catechols can be separated from noncatechols by adsorption of the former on alumina.

This communication presents evidence, obtained when this method was used to separate metabolites of L-dopa-^{14}C, that the glucuronides of the amines, 3,4-dihydroxyphenylethylamine (dopamine) and 3-methoxy-4-hydroxyphenylethylamine (3-*O*-methyldopamine), are retained by Dowex-50 in this separation and are eluted by the neutral buffer. These amine glucuronides are not catechols, because conjugation with glucuronic acid occurs in position 4;[7,8] therefore, they both subsequently appear in the effluent during the alumina separation of the amino acids, and they thus contribute to a fraction normally considered to consist only of 3-methoxy-4-hydroxyphenylalanine (3-*O*-methyldopa).

L-Dopa-3-^{14}C (5 μc; 192 μg) was injected into the isolated perfused rat liver system as described previously.[5] In some experiments, solutions containing 2 mg of the monoamine oxidase inhibitor, β-phenylisopropylhydrazine (JB516), or 6·7 mg of the L-aromatic amino acid decarboxylase inhibitor, *N*-(DL-seryl)-*N'*-(2,3,4-trihydroxybenzyl)-hydrazine (RO 4-4602), were added to the perfusate at 5 or 30 min before the dopa-^{14}C. Livers were perfused for 30 or 300 min. Bile was collected continuously. At the end of the perfusion, the liver was frozen in crushed dry ice.

Frozen livers were homogenized in 5 vol. of ice-cold 0·4 M perchloric acid; after centrifugation, 1·0 ml of a 1% solution of disodium ethylenediaminetetraacetate (EDTA) was added to 10 ml of the supernatant. Bile (1·0 ml) was treated with 0·1 ml of 1% EDTA and 4·4 ml of 0·4 M perchloric acid, and precipitated proteins were removed by centrifugation. Perchlorate was removed by adjustment of the ice-cold solution to pH 5 with KOH and centrifugation.

Amines and amino acids were isolated by the method of Bartholini and Pletscher[2] using Dowex-50W × 4 columns (diameter, 0·5 cm; height, 5 cm; Na^+ form; 200–400 mesh). Protein-free extracts were adjusted to pH 2, or sometimes to pH 4, before application to the columns. Amino acids were eluted with 0·5 M potassium acetate (pH 6·5) or 0·5 M ammonium formate (pH 6·5), and then amines were eluted with 2 N HCl. Catechols were separated from noncatechols by alumina adsorption at pH 8·4, followed by elution with 0·2 N HCl.

Radioactivity in aliquots of each fraction and in aliquots of liver homogenate and of bile was measured by addition to a 2:1 mixture of toluene–PPO–DMPOPOP* and Triton X-100 and counting in a liquid scintillation counter.

Methanol–acetone extracts of bile[9] were applied to 12 × 12-in. sheets of Whatman 3 MM paper, and two-dimensional chromatograms were developed (isopropanol–water–ammonia (20:1:2) for 20 hr followed by *n*-butanol–acetic acid–water (12:3:5) for 8 hr). Aliquots of the amino acid fraction eluted from Dowex-50 with ammonium formate were lyophilized, and the residue was dissolved in a small volume of 0·01 N HCl and co-chromatographed with reference standards in *n*-butanol–acetic acid–water.

Distribution of radioactivity on strip chromatograms was determined by using a 4 π gas-flow strip scanner. Autoradiograms were made by exposing x-ray film (Kodak no screen) to the two-dimensional chromatograms for 3 weeks.

In some experiments, 10-ml aliquots of the potassium acetate eluate from Dowex-50 were adjusted to pH 5·4 and incubated at 37° for 18 hr with Glusulase (Endo Laboratories; 0·1 ml, containing

* PPO = 2,5-diphenyloxazole; DMPOPOP = 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

10,000 units of β-glucuronidase and 5000 units of sulfatase). Other aliquots of this eluate were adjusted to pH 6·5 and incubated with 20 mg of β-glucuronidase (Sigma Chemical Company; 1000 units). Parallel incubations of aliquots of the eluates were carried out without enzymes. Protein (of the enzyme) and perchlorate were again precipitated, and the extracts were either concentrated for paper chromatographic studies or metabolites were separated by Dowex-50 and alumina chromatography.

So that the glucuronides of dopamine and 3-*O*-methyldopamine could be obtained in amounts adequate for a study of their chromatographic and fluorometric properties, carrier dopa (10 mg) was mixed with the radioactive substrate in one perfusion. The glucuronides of dopamine and of 3-*O*-methyldopamine were then isolated from liver by Dowex-50, alumina, and paper chromatography procedures. It was not possible, however, to separate the amine glucuronides from each other in these experiments. It was assumed that the specific activity of the glucuronides was the same as that of the precursor dopa.

At 30 min after the injection of L-dopa-^{14}C, 31·6 and 30·4 per cent of the dose was in the liver in two control experiments, whereas only 9·4 per cent was in the liver in one perfusion in which RO 4-4602 was added to the perfusate. In control livers, most of the ^{14}C was in the noncatechol fraction of the potassium acetate eluate, a fraction which usually is considered to consist of 3-*O*-methyldopa only (Table 1). In the perfusion to which RO 4-4602 was added, considerable radioactivity was present also in the catechol amino acid fraction.

TABLE 1. SEPARATION OF METABOLITES OF L-DOPA-^{14}C IN PROTEIN-FREE EXTRACTS OF ISOLATED LIVERS AFTER 30 MIN OF PERFUSION

	% of total ^{14}C in liver*					% of total ^{14}C in noncatechol "amino acid" fraction in liver perfusion 1*	
	No RO 4-4602			RO 4-4602 in perfusate		No RO 4-4602	
Fraction	pH 2†	pH 2†	pH 4†	pH 2†	pH 4†	pH 2†‡	pH 2†§
Dowex effluent	21·0	19·5	28·1	7·1	96·2	4·6	6·5
Potassium acetate eluate							
Total "amino acids"	74·0	74·4	68·8	87·4	4·3	96·2	4·1
Noncatechol	72·8	73·9		34·6		96·2	2·0
Catechol	1·3	1·4		50·2		0	2·0
2 N HCl eluate							
Total amines	1·8	1·0	1·4	0·9	0·3	1·7	90·8
Noncatechol	0·8	0·3		0·4		1·1	43·0
Catechol	1·2	0·6		0·6		0·5	47·9

* Values are means of duplicate experiments.

† pH of extract added to Dowex-50 column.

‡ Aliquots of noncatechol "amino acid" fraction were incubated without Glusulase preparation (blank) before separation of metabolites.

§ Aliquots of noncatechol "amino acid" fraction were incubated with Glusulase preparation before separation of metabolites.

Paper chromatographic studies of the presumptive amino acid fraction from Dowex-50 showed that, in the perfusion to which RO 4-4602 had been added, dopa-^{14}C and 3-*O*-methyldopa-^{14}C were present, while in the two control perfusions these compounds were not detected and a radioactive compound with a slower R_f in *n*-butanol–acetic acid–water was abundant (Fig. 1).

When the noncatechol "amino acid" fraction from control liver 1 was incubated with Glusulase and fractionated by column chromatography, almost equal amounts of ^{14}C were detected in the catechol and noncatechol amine fractions (Table 1). In the blank reaction mixture (incubated without enzyme), most of the radioactivity was found in the noncatechol "amino acid" fraction, as previously. Paper chromatographic studies after β-glucuronidase incubation of this fraction showed clearly that the products were dopamine-^{14}C and 3-*O*-methyldopamine-^{14}C. The amines were found in approximately equal amounts in liver perfusion 1, but in perfusion 2 almost three times as much 3-*O*-methyl-

dopamine-^{14}C as dopamine-^{14}C was detected (Fig. 1). Complete hydrolysis both by Glusulase and by β-glucuronidase indicated that the compounds were glucuronides and not sulfates. These results show that in control liver perfusions the potassium acetate eluate from Dowex-50 had contained the glucuronides of dopamine-^{14}C and 3-*O*-methyldopamine-^{14}C and not 3-*O*-methyldopa-^{14}C.

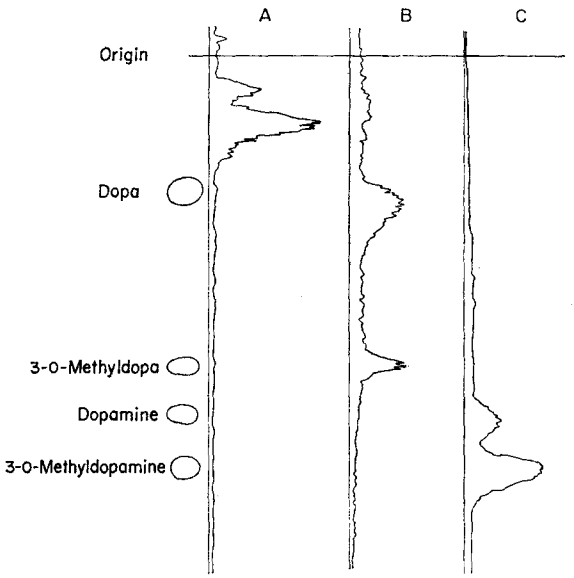

FIG. 1. Radioactivity on one-dimensional chromatograms (*n*-butanol–acetic acid–water; 12:3:5) of "amino acid" fraction (eluted from Dowex-50 columns with 0·5 M ammonium formate). Locations of co-chromatographed standards (spray reagent: diazotized *p*-nitroaniline) are shown at left. (A) Control perfused rat liver 2; (B) perfused liver with L-aromatic amino acid decarboxylase inhibitor (RO 4-4602) added to perfusate; (C) "amino acid" fraction of extracts of control perfused rat liver 2, after incubation with β-glucuronidase.

The recovery of 3-*O*-methyldopa through enzyme incubation and column separations was determined. 3-*O*-methyldopa (10 μg) was incubated with Glusulase or β-glucuronidase and the products were fractionated by Dowex-50 (addition at pH 2) and alumina chromatography. The recoveries of 3-*O*-methyldopa in the noncatechol amino acid fraction (determined by the fluorometric method of Sharpless *et al.*[10]) were 84·6 and 88·8 per cent after Glusulase incubation, and 98·9 and 103·9 per cent after glucuronidase incubation. When the recovery through the column separation was determined using 3-*O*-methyldopa-U-^{14}C (New England Nuclear) (140 ng, 0·27 μc), 95·2 and 94·2 per cent of added radioactivity was recovered in the noncatechol "amino acid" fraction.

Additional evidence that some compound other than 3-*O*-methyldopa was present in the noncatechol "amino acid" fraction came from separations, on Dowex-50 and alumina, of the metabolites of L-dopa-^{14}C in extracts of bile from five rat liver perfusions in which JB516 was added to the perfusate. In these perfusions, 27·3–48·3 per cent of injected radioactivity was excreted in bile. Most of this ^{14}C (79·9 per cent ± 2·4, S.E.) was found in the effluent of the Dowex-50 column, but considerable amounts (8·1 per cent ± 1·2, S.E.) were in the noncatechol "amino acid" fraction also. However, two-dimensional chromatograms with added standard showed clearly that, as in control perfusions without JB516,[5] no 3-*O*-methyldopa-^{14}C was detectable in bile. Elution of radioactive areas from these chromatograms showed that amounts of ^{14}C recovered as the glucuronide of 3-*O*-methyldopamine were comparable to those found in the noncatechol "amino acid" fraction.

In the isolation of metabolites of ^{14}C-dopa, serious error could result by attributing radioactivity in the noncatechol "amino acid" fraction solely to 3-*O*-methyldopa-^{14}C, since amine glucuronides also appear in this fraction. It is not known whether amine sulfates would also contribute to this fraction; these metabolites were not detected in bile, plasma, or liver in our perfusions.

The amine glucuronides can be separated from 3-*O*-methyldopa by paper chromatography (Fig.1), but other attempts to separate them were largely unsuccessful. Variation of the pH of the solution

added to the Dowex-50 columns effected only partial separation. At pH 5, neither the amine glucuronides nor 3-*O*-methyldopa was retained by the resin. At pH 4, the glucuronides were almost quantitatively retained by the resin and eluted with potassium acetate (Table 1). Although it has been shown previously[11] that dopa applied to Dowex-50 at pH 4 is not retained and is quantitatively recovered in the effluent, we found that when 53 ng (0·1 μc) of 3-*O*-methyldopa-^{14}C was added to Dowex-50 at pH 4 only 61·4 per cent of the radioactivity was in the effluent and 36·3 per cent was retained by the resin and was eluted by the potassium acetate. An attempt was made to separate these compounds by using a carboxylic acid cation-exchange column which does not retain amino acids. When 2 ml solution containing 145 nmoles 3-*O*-methyldopamine glucuronide and 36 nmoles dopamine glucuronide was added at pH 6·1 to a column of Amberlite CG 50 (diameter, 7 mm; height, 10 mm; 200–400 mesh) equilibrated to pH 6·1,[12] 94 per cent of the radioactivity was recovered in the effluent. Further studies are needed to determine if the amine glucuronides can be separated from 3-*O*-methyldopa by gel filtration techniques.

Initially it was considered unlikely that the presence of glucuronides of dopamine or of 3-*O*-methyldopamine would interfere in the fluorometric determination of 3-*O*-methyldopa. When a series of solutions containing different concentrations of the glucuronides of dopamine and 3-*O*-methyldopamine in the proportions of 1:4 were oxidized under the standard conditions for measurement of 3-*O*-methyldopa concentration,[10] the fluorescence readings of the reversed blanks were always approximately twice as high as those of the reagent blank. However, a significant fluorescence, equivalent to between 9 and 10 per cent of that produced by equimolar solutions of 3-*O*-methyldopa, was developed by the amine glucuronides. It is likely that all of the glucuronide fluorescence was contributed by the glucuronide of dopamine, because this compound has a free phenolic group. It can be suspected, therefore, that this compound would give a considerable interference in the fluorometric measurement of 3-*O*-methyldopa if it were present in the same solution. However, the determinations that we have previously reported[10] of 3-*O*-methyldopa in plasma from patients with Parkinson's disease who were receiving L-dopa are unlikely to be in significant error because the amounts of conjugated dopamine in plasma of such patients have been shown to be very small.[13]

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