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DEVELOPMENT OF A STANDARDIZED ANALYSIS STRATEGY FOR BASIC DRUGS, USING ION-PAIR EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

VI. DRUG LEVEL DETERMINATION IN SALIVA

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SUMMARY

Assay methods for measuring saliva levels of carbamazepine and its active metabolite, and of amidopyrine are developed using a standardized analysis strategy. Both assay methods include ion-pair extraction of the analytes with octylsulphate as the counter-ion and chromatography on a CN-bonded phase using a hexane—dichloromethane—acetonitrile—propylamine mixture as the mobile phase. Both methods are applied to patient samples.

INTRODUCTION

It has been suggested that saliva might be substituted for plasma in therapeutic drug monitoring and pharmacokinetic studies. Observations and considerations supporting this suggestion include the following. (1) It has been established that for a number of drugs the saliva concentration is proportional to the plasma concentration. (2) For some drugs it has been shown that the saliva concentration is equal to the free drug concentration in plasma, i.e. the pharmacologically active portion of the total drug concentration in plasma. (3) Saliva is readily available and can be collected by non-invasive techniques, which is of great practical importance particularly for paediatric, geriatric and out-patients.

The advantages and possibilities of drug level determinations in saliva have been reviewed by Danhof and Breimer [1] and by Horning et al. [2]. From these reviews it appears, however, that extensive research is still needed before the suggested substitution of saliva for plasma could be entirely

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justified. This implies, among others, drug level determinations in an extensive number of samples and for a wide variety of drugs. It could therefore be advantageous to use a standardized analytical procedure which is applicable to a large number of drugs and requires only minor modifications in order to make the assay method selective for the drug under investigation. In our laboratory such a standardized analysis strategy has been developed for basic drugs. Its philosophy and advantages [3, 4] as well as its application to the analysis of pharmaceutical dosage forms [5, 6] and cosmetics [7] have been reported previously. It is the aim of the present paper to demonstrate the usefulness of the strategy for drug level determinations in saliva. This will be done by constructing a method for the determination of carbamazepine and its active metabolite and for amidopyrine.

EXPERIMENTAL

Apparatus

A Varian 8500 liquid chromatograph was used, equipped with a Valco loop injector (sample loop volume 100 μl), a fixed-wavelength (254 nm) ultraviolet (UV) detector, a Varian 9176 recorder and a Varian Vista CDS 401 chromatographic data system. Chromatography was performed on a MicroPak CN-10 column (particle size 10 μm), 300 × 4 mm (Varian, Palo Alto, CA, U.S.A.). The characteristics of the briefly used LiChrosorb CN column were: particle size 10 μm, dimensions 250 × 4 mm (E. Merck, Darmstadt, F.R.G.).

Chemicals and reagents

Sodium *n*-octylsulphate (for tenside tests) was purchased from Merck. *n*-Hexane, dichloromethane and acetonitrile were HPLC grade and purchased from Merck or Fluka (Buchs, Switzerland). All other reagents were analytical reagent grade and obtained from Merck, except propylamine which was purchased from Fluka. Carbamazepine and carbamazepine-10,11-epoxide were kindly supplied by Ciba-Geigy, and amidopyrine was a kind gift from Hoechst.

Collection of saliva samples

Method construction was performed using saliva samples from different donors not undergoing drug therapy. Only whole saliva, obtained without stimulation of the salivary flow, was used. Samples of patients and volunteers were obtained in the same way. No special precautions concerning, for example, beverage and food intake of the donors were taken. The samples were briefly centrifuged before sample preparation in order to precipitate debris.

Extraction procedure

To 1 ml of saliva standard or sample, 100 μl of internal standard solution were added in glass centrifuge tubes equipped with PTFE-covered screw-caps. After vortexing, 10 ml of 0.05 *M* sodium *n*-octylsulphate solution in phosphate buffer pH 3.0 (I = 0.4) were added. The mixture was homogenized by vortexing, then 5 ml of chloroform were added. Partitioning was performed by gently shaking the tubes longitudinally in a shaking bath for 30 min (vortexing

for a few minutes might be equally efficient but was not investigated). After centrifugation, the aqueous layer was discarded and 4 ml of the organic solvent were transferred to a clean vial with conical bottom and evaporated to dryness under a gentle nitrogen stream at ± 40° C. Each extract was reconstituted just prior to chromatography with 200 μl of dichloromethane; 100 μl were injected.

The extraction of plasma was also carried out using the method described above. The plasma proteins, however, were precipitated with 2 ml of acetonitrile, the supernatant was transferred to a new centrifuge tube and the acetonitrile was evaporated in a water bath, before addition of the counter-ion solution [8].

Assay standards

Assay standards were prepared by spiking drug-free saliva or plasma with 100 μl of an aqueous solution of the analytes to give the desired concentrations. The stock solution of carbamazepine and carbamazepine-10,11-epoxide was prepared by dissolving the drugs in a few millilitres of acetonitrile and then bringing to volume with double-distilled water.

Internal standard solutions

The internal standard solution for the carbamazepine assay contained 1.0 μg of nitrazepam per 100 μl of water. A fresh solution was made each day. The internal standard solution for the amidopyrine assay contained 500 ng of promethazine · HCl per 100 μl of water.

Recovery

The overall recoveries were established by interpolation of the peak area of the extracted analytes on a calibration curve (peak area versus concentration) of standards prepared in dichloromethane.

Quantitation

The peak area ratio of analyte to internal standard was used as quantitation criterion. The peak area ratios were plotted against concentration to obtain standard calibration curves. A new calibration curve was made with each sample set.

RESULTS AND DISCUSSION

The standardized analysis strategy [3—8] consists of an ion-pair extraction step using chloroform as the solvent and either sodium *n*-octylsulphate (NaOS) at pH 3.0 or di-(2-ethylhexyl)phosphoric acid (HDEHP) at pH 5.5 as ion-pairing reagents, followed by direct HPLC analysis of the extract using a CN-bonded phase with either hexane—dichloromethane—acetonitrile—propylamine (50:50:25:0.1) or acetonitrile—water—propylamine (90:10:0.01) as standardized eluent. Selectivity of the method for a particular analysis problem is obtained by optimizing the volume ratio of the mobile phase components. The polarity of the analyte usually determines which of the counter-ions and which of the eluents should be used, although other factors such as the opportunity of detection at wavelengths in the lower UV region might

influence the choice of eluent. In the present study, a fixed-wavelength (254 nm) detector was used, ruling out this possibility, but even then sensitivity aspects (see, for example, the carbamazepine assay) might influence the eluent choice. As was demonstrated previously [3, 5], HDEHP is a more powerful ion-pairing reagent than NaOS and should therefore be preferred. However, in the present work all extractions are performed using NaOS extraction to demonstrate that this reagent also allows high extraction yields. Furthermore, it should be noted that due to the low pK_a (5.0) of aminopyrine, no ion-pair formation with HDEHP at pH 5.5 would be possible. The foregoing remarks demonstrate the need for flexibility in a standardized analysis scheme, and although both the stationary and the mobile phases, the extraction solvent, the pH, and ion-pairing reagents are standardized, care has been taken during the conception and development of the strategy to allow enough versatility to be able to meet certain special needs in a particular analysis problem.

Carbamazepine

Since at 254 nm normal-phase chromatography affords higher sensitivity than the reversed-phase mode [9], the carbamazepine assay method was developed by use of the hexane—dichloromethane—acetonitrile—propylamine eluent. The original (50:50:25:0.1) volume ratio was optimized by halving the dichloromethane content in order to separate carbamazepine and its active metabolite, carbamazepine-10,11-epoxide, and to resolve both compounds from caffeine and other sample components. At a flow-rate of 2 ml/min, caffeine, carbamazepine and carbamazepine-10,11-epoxide gave retention times of 4.4, 6.7 and 11.5 min, respectively. Several drugs were chromatographed to test for interference and for use as internal standard. Their retention times relative to carbamazepine are listed in Table I. Nitrazepam was selected as internal standard. None of the drugs investigated interfered with either

TABLE I

RETENTION TIMES, RELATIVE TO CARBAMAZEPINE, OF VARIOUS DRUGS

Drug	Relative retention time
Thioridazine	0.65
Nitrazepam	0.75
Amidopyrine	0.83
Procaine	0.85
Carbamazepine*	1.00
Phenylbutazone	1.28
Mesoridazine	1.33
Carbamazepine-10,11-epoxide	1.72
Antipyrine	2.15
Chlordiazepoxide	2.28
Primidone	3.40
Phenobarbital	3.40
Paracetamol	3.87
Diphenylhydantoin	4.45
Quinidine	>5
Procainamide	>5

*Retention time = 6.7 min.

carbamazepine, carbamazepine-10,11-epoxide or nitrazepam. Since large intra- and inter-individual differences in UV-absorbing saliva constituents have been observed [10] the method was applied to a large number of saliva samples from various donors, collected at different times of day. No interference from endogenous compounds was observed. The analytical recoveries of carbamazepine and its 10,11-epoxide from both saliva and plasma were assessed at the 1 μg/ml level using the octylsulphate extraction technique. The values obtained are presented in Table II. As would be expected the recoveries are comparable for both analytes and are higher from saliva than from plasma. It should be noted that storage of the evaporated extracts at 4°C during 24 and 48 h results in losses of both analytes of about 10% and 25%, respectively.

TABLE II

PERCENTAGE OVERALL RECOVERY FOR CARBAMAZEPINE AND CARBAMAZEPINE-10,11-EPOXIDE FROM SALIVA AND PLASMA

Samples of 1 μg/ml for each drug were used; $n = 6$.

Sample	Percentage recovery	
	Carbamazepine	10,11-Epoxide
Saliva	95.8 ± 2.0%	96.8 ± 2.9%
Plasma	87.0 ± 3.2%	89.3 ± 3.8%

The linearity of the calibration curves in both saliva and plasma was evaluated for carbamazepine in the 0.1—10.0 μg/ml concentration range and for the 10,11-epoxide in the 0.1—5.5 μg/ml range. The standard curves were rectilinear in the range tested for both analytes and both matrices (for carbamazepine mean $r^2 = 0.999$, for the 10,11-epoxide mean $r^2 = 0.998$). A chromatogram of a saliva standard is shown in Fig. 1. The within-day precision of the assay method was evaluated at the 0.5 μg/ml saliva and 3.0 μg/ml saliva levels by analysing replicate spiked samples ($n = 6$). Coefficients of variation of 4.2% and 2.9%, respectively, for carbamazepine and 4.9% and 3.7%, respectively, for the epoxide metabolite were found. The limits of detection at a signal-to-noise ratio of 3 were estimated to be 0.004 μg of carbamazepine per ml of saliva and 0.040 μg of carbamazepine-10,11-epoxide per ml of saliva.

In order to demonstrate the usefulness of the method it was applied to saliva samples of patients treated with Tegretol®. Although most of these patients received various other drugs as well, no interference was observed. The purity of the extracts is demonstrated in Fig. 2, which shows a chromatogram obtained from a patient treated with Tegretol (200 mg, four times a day) and Largactil® (25 mg, three times a day). The sample was collected 1 h after the last tablet intake and carbamazepine and 10,11-epoxide levels were found to be 4.17 and 0.20 μg/ml, respectively. The chromatogram also shows an unidentified peak with a retention time of 2.5 min. It is interesting to note that this peak was only present in saliva from patients on carbamazepine and that the area of this peak increased with increasing carbamazepine and 10,11-epoxide levels. It might therefore be a metabolite of carbamazepine. It can be concluded that the method is very useful for assaying carbamazepine and its

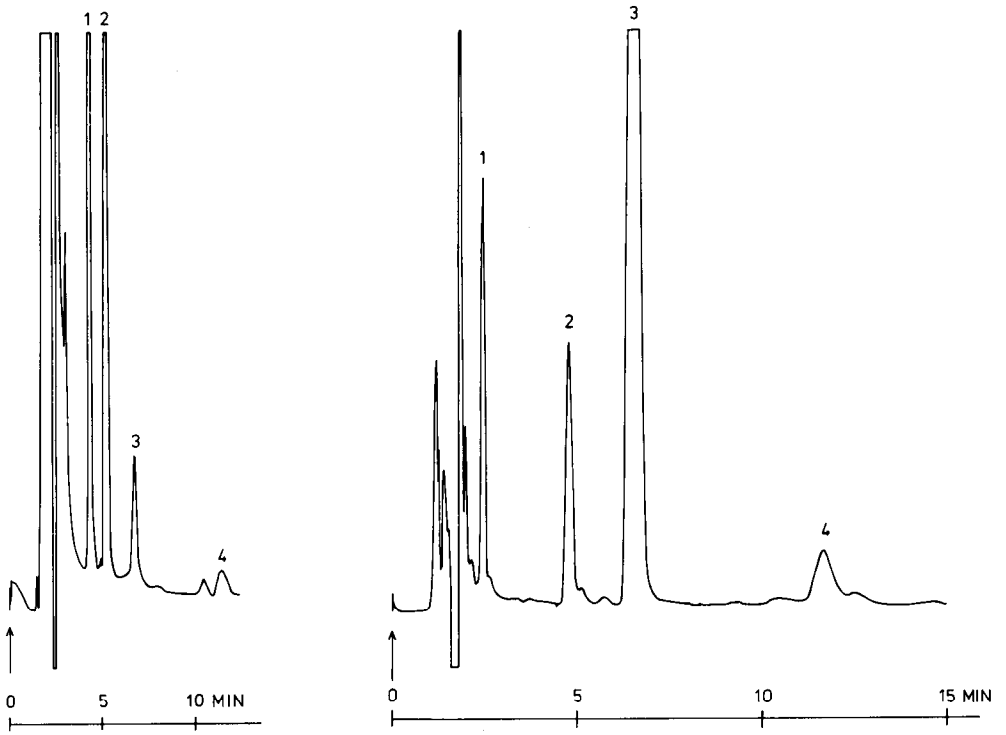


Fig. 1. Chromatogram of a spiked saliva extract. Peaks: 1 = caffeine, 2 = nitrazepam (internal standard), 3 = carbamazepine (106 ng/ml), 4 = carbamazepine-10,11-epoxide (102 ng/ml). Column: MicroPak CN-10 (10 μm, 300 × 4 mm). Mobile phase: *n*-hexane—dichloromethane—acetonitrile—propylamine (50:25:25:0.1). Flow-rate: 2 ml/min. Detection sensitivity: 0.01 a.u.f.s.

Fig. 2. Chromatogram of a saliva sample from a patient treated with Tegretol and Largactil. Peaks: 1 = unknown substance, probably a metabolite, 2 = caffeine, 3 = carbamazepine (4.17 μg/ml), 4 = carbamazepine-10,11-epoxide (0.20 μg/ml). The chromatographic conditions are the same as in Fig. 1, except detector sensitivity which was 0.02 a.u.f.s.

epoxide metabolite in saliva. It also should be valuable for assaying the drugs under study in paired samples of saliva and plasma in order to elucidate further the relationship between saliva and plasma levels of both carbamazepine and its epoxide metabolite.

We were able to find only one HPLC method designed for measuring carbamazepine levels in saliva. This method, reported by Westenberg et al. [11], has proved very valuable since it allowed the existence of a correlation to be established between carbamazepine levels in plasma and saliva. However, although the method has been applied to a large number of saliva samples from seven patients receiving carbamazepine as long-term medication, it did not allow the detection of the 10,11-epoxide metabolite in saliva. This is certainly due to the rather low sensitivity (0.4 μg/ml) for the metabolite. Our HPLC method, which is sensitive to 40 ng of 10,11-epoxide per ml, revealed the presence of the 10,11-epoxide in every saliva sample from patients receiving Tegretol, analysed so far.

Amidopyrine

The hexane—dichloromethane—acetonitrile—propylamine eluent was also used for the determination of amidopyrine in saliva and plasma. Optimization of the volume ratio of the mobile phase constituents was done in parallel on a LiChrosorb CN column and on a MicroPak CN column. Volume ratios of 70:25:5:0.1 and 25:50:25:0.1, respectively, giving capacity factors of 4.3 and 4.8, respectively, were found to be appropriate with respect to peak shape, retention, analysis time and interference from sample constituents and possible co-administered drugs. The substantial difference between both volume ratios once again shows that (1) considerable differences between CN columns of different manufacturers exist, although both columns are packed with the same packing material, and (2) brand-to-brand and lot-to-lot differences do not jeopardize the standardized strategy since the volume ratio of the mobile phase constituents can easily be adapted to the particular column and analysis problem.

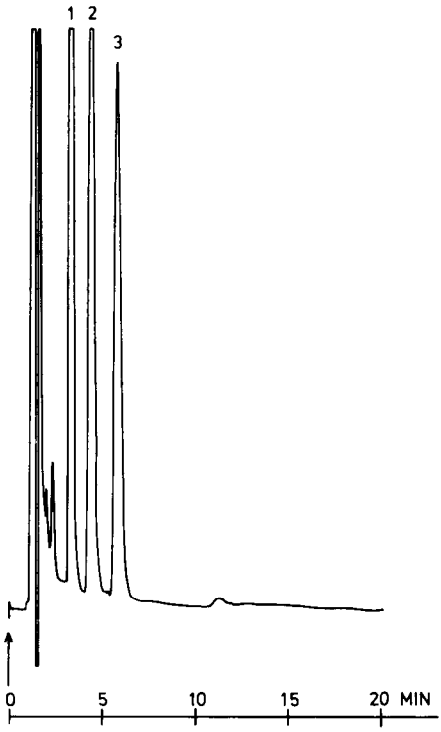
Although both columns combined with the appropriate mobile phase were equally efficient for the determination of amidopyrine in saliva and plasma, the MicroPak-CN column combined with hexane—dichloromethane—acetonitrile—propylamine (25:50:25:0.1) was chosen for subsequent use. This choice, however, is arbitrary. The retention times relative to amidopyrine of a number of drugs chromatographed to test for interference and for use as internal standard, are tabulated in Table III. Promethazine was chosen as internal standard.

TABLE III

RETENTION TIMES, RELATIVE TO AMIDOPYRINE, OF VARIOUS DRUGS

Drug	Relative retention time
Diazepam	0.38
Propyfenazone	0.40
Triflupromazine	0.50
Chlorimipramine	0.60
Amitriptyline	0.66
Procaine	0.73
Promethazine	0.74
Thioridazine	0.79
Imipramine	0.81
Amidopyrine*	1.00
Mesoridazine	1.55
Antipyrine	1.88
Diphenylhydantoin	2.17
Nortriptyline	2.24
Paracetamol	3.86
Primidone	3.97
Procainamide	>5
Phenobarbital	>5
Phenylbutazone	>5
Lidocaine	>5

*Retention time = 4.5 min.



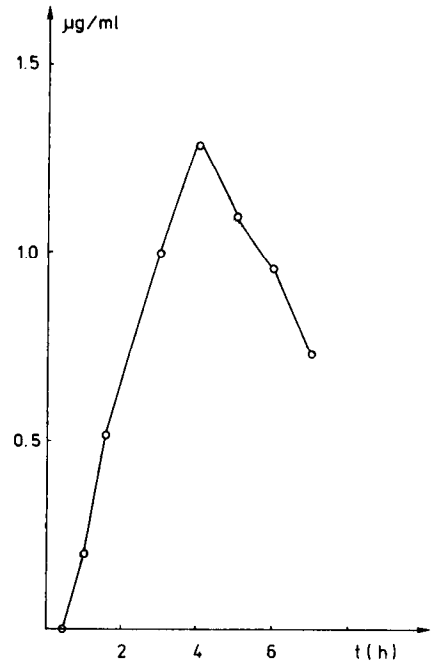


Fig. 3. Chromatogram of a spiked saliva extract. Peaks: 1 = caffeine, 2 = promethazine (internal standard), 3 = amidopyrine (1 µg/ml). Column: MicroPak CN-10 (10 µm, 300 × 4 mm). Mobile phase: *n*-hexane—dichloromethane—acetonitrile—propylamine (25:50:25:0.1). Flow-rate: 2 ml/min. Detector sensitivity: 0.02 a.u.f.s.

Fig. 4. Saliva concentration—time profile of a volunteer who ingested 300 mg of amidopyrine.

The analytical recoveries of amidopyrine from both saliva and plasma were assessed in the 1—3 µg/ml range. The mean values obtained were 94.7 ± 3.9% and 93.9 ± 4.2%, respectively (n = 7). Here also losses of about 10% occur when the evaporated extracts are stored at 4° C for 24 h. The calibration curves in both saliva and plasma were rectilinear up to at least 5 µg/ml (mean r^2 = 0.998). A chromatogram of a saliva standard is shown in Fig. 3. The within-day precision was evaluated at the 200 ng/ml and 3.0 µg/ml levels by analysing replicate spiked samples (n = 6). Coefficients of variation of 5.8% and 3.4%, respectively, were found, and the detection limit at a signal-to-noise ratio of 3 was estimated to be 20 ng/ml of saliva.

In order to demonstrate the usefulness of the method it was applied to a study of the pharmacokinetics of amidopyrine in one subject. The volunteer was given a single oral dose of 300 mg of amidopyrine; saliva samples were collected 0.5, 1, 1.5, 3, 4, 5, 6 and 7 h after ingestion of the cachet. The results are reported in Fig. 4 as a saliva concentration—time profile. It can be seen that the peak level is reached between 3 and 5 h after ingestion.

CONCLUSION

It can be concluded that the standardized analysis strategy which was previously shown to be applicable to pharmaceutical dosage forms and cosmetics, is also applicable to drug-level determinations in saliva. This was exemplified for carbamazepine and its active metabolite, on the one hand, and amidopyrine on the other.

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