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Determination of total dopamine, *R*- and *S*-salsolinol in human plasma by cyclodextrin bonded-phase liquid chromatography with electrochemical detection☆

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Abstract

A reliable and sensitive high-performance liquid chromatographic (HPLC) method is presented for the determination of total (free and conjugated) plasma dopamine and the enantiomers *R*- and *S*-salsolinol. Plasma is purified on two cartridges, containing primary and secondary amines and phenylboronic acid. Dopamine, *R*- and *S*-salsolinol are then separated by HPLC using a β-cyclodextrin-OH phase column. The eluate is monitored electrochemically, without further purification nor derivatization. The method is suited for routine analysis. It allows the detection of total (free and conjugated) dopamine and *R*- and *S*-salsolinol in human plasma in concentrations as low as 0.02 ng/ml plasma. The sensitivity is sufficient to measure the naturally occurring levels of salsolinol.

1. Introduction

Various quantitative methods, such as radioenzymatic determinations, gas chromatography and in particular liquid chromatography, are used for the analysis of salsolinol (SAL), and dopamine in body fluids and tissues [1–6]. All these methods require an extensive clean-up procedure, sometimes followed by concentration of the sample necessary to obtain accurate and reliable results. The chromatographic methods, although very useful, are rather laborious and can not be easily automated, which is a great disadvantage when many samples have to be analyzed. For plasma and urine samples far too many endogeneous substances, interfering with analysis of the catecholamines, are co-extracted by *n*-butanol, a commonly used solvent.

Much of the criticism concerning reports on the detection of SAL or dopamine in mammalian tissues and fluids has been directed at the analytical methods used. In all papers mentioned above, total (R + S) concentrations of SAL in plasma or urine were measured. Since the two enantiomers of SAL do not exert identical biological activities, a new method should be able to determine the enantiomeric composition of endogeneous SAL in human plasma. Two groups have determined the composition of the enantiomers of SAL in urine samples using a derivatization step [7,8].

The main objective of the present study was to

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☆ Dedicated to Prof. Arnold Brossi on the occassion of his 70th birthday.

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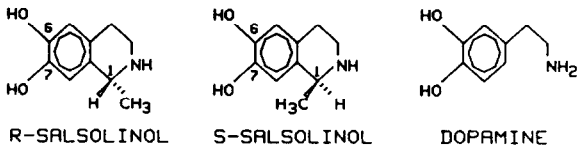

Fig. 1. Structures of *R*- and *S*-enantiomers of salsolinol and dopamine.

elaborate a method to measure simultaneously the enantiomers of salsolinol and dopamine in human plasma. The extraction procedure used is fast, efficient and free from artifactual formation of SAL despite the high dopamine concentrations in the sample.

Salsolinol, (6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline) can be formed by condensation of dopamine either with acetaldehyde or with pyruvic acid followed by decarboxylation (Fig. 1). Salsolinol has been detected in human urine, cerebrospinal fluid, brain tissue as well as in human plasma. Differences in urinary excretion of salsolinol have been found between healthy volunteers and untreated parkinsonian patients as well as alcoholics. In healthy subjects the *R*-enantiomer predominates in urine samples, while in alcoholics the *S*-enantiomers was found to be predominant [9–12].

2. Experimental

2.1. Apparatus

The high-performance liquid chromatographic system used consisted of a Knauer constant-flow pump (type 364.00, Berlin, Germany) equipped with an additional diaphragm pulse-damper (ESA0133, Bedford, MA, USA), a Spectra-Physics autosampler (SP 8780XR, San Jose, CA, USA), a Coulochem detector (ESA 5100A) with a high-sensitivity analytical cell (ESA 5011) and a recorder (Chromatopac C-R3A, Shimadzu, Kyoto, Japan), and a stainless-steel column 200 × 8 × 4 mm I.D. packed with Nucleodex β-OH, 5 μm (Macherey-Nagel, Düren, Germany).

2.2. Reagents and standards

All inorganic reagents were of analytical grade and used without any further purification. Methanol was obtained from Merck (Lichrosolv-grade). Milliporegrade water was used for preparation of reagents and solutions.

Stock solutions of dopamine (177.7 μg/ml), and racemic *R*- and *S*-salsolinol (414.93 μg/ml) were prepared by dissolving 3.78 mg of dopamine hydrochloride and 10 mg of *R*- and *S*-salsolinol hydrochloride in 20 ml of 0.1 *M* perchloric acid, respectively. These standards are stable at −20°C for at least 6 months. The working standard used was equivalent to 3.54 ng/ml of dopamine and 8.30 ng/ml of *R*- and *S*-salsolinol in native plasma. Dopamine-HCl, *R*- and *S*-salsolinol-HCl were obtained from Sigma (St. Louis, MO, USA). *S*-salsolinol-HBr was a gift from Dr. Dostert (Farmitalia Carlo Erba, Milan, Italy).

The mobile phase used for HPLC separation of the catecholamines consisted of a solution containing 1% TEA (triethylamine), 2% methanol and 250 mg of 1-octanesulfonic acid per liter of freshly distilled water. The pH of the solution was adjusted to 3.9 with acetic acid.

All other chemicals used were obtained either from Merck (Darmstadt, Germany) or Fluka (Neu-Ulm, Germany).

The solid-phase extraction columns used were a Varian Bond Elut primary/secondary amine (PSA) column and a phenylboronic acid (PBA) column (Varian, Harbo City, USA).

2.3. Collection and pretreatment of blood samples

Blood samples (10 ml) were collected directly into polyethylene tubes containing 0.08 ml of 1.5% EDTA per ml blood and centrifuged for 15 min at 200 *g* followed by 10 min at 2000 *g*; the plasma was removed and added to a preservative of 0.4 *M* perchloric acid containing 0.05% ascorbic acid and 0.02% semicarbazide (3:1, v/v). The samples were stored in the dark at −80°C.

All glassware was pretreated with perchloric acid to avoid binding of the amines on the glass surface.

Before analysis the samples were thawed, ascorbic acid (3 μg/ml) and strong (60%) perchloric acid (75 μl/ml) were added under shaking and the samples centrifuged for 10 min at 2000 *g* to remove solids and proteins. For each preparation approximately 3 ml plasma were used.

2.4. Hydrolysis

The sulfoconjugated catecholamines, *R*- and *S*-salsolinol were preserved with ascorbic acid and EDTA and hydrolyzed by boiling with acid for 20 min. After cooling (at room temperature) the solution was brought to pH 5 with sodium-hydroxide (containing EDTA) and lyophilized.

2.5. Extraction

For the selective liquid–solid extraction of *R*- and *S*-salsolinol and dopamine PSA and PBA cartridges were used in series. The cartridges were conditioned prior to application of the sample by washing sequentially first only the PSA cartridge with 1 ml of methanol and 1 ml of 0.1 *M* HCl, then both cartridges with 1 ml of methanol, 2.5 ml of 0.3% ammonium hydroxide and 5 ml of 5 m*M* phosphate buffer (pH 8.5). Then the sample (dissolved in 1 ml of water) buffered at pH 5 ± 0.5 was loaded on the top (PSA), and passed through the cartridge by gentle low pressure aspiration. After 4 washings (2.5 ml each) with 5 m*M* phosphate buffer (pH 8.5) and subsequently with 1 ml methanol, the adsorbed catecholamines were eluted from the PBA cartridge with 1 ml of 0.1 *M* HCl. An aliquot of 40 μl was then directly applied to the HPLC column.

2.6. HPLC separation and detection

Analyses of dopamine and salsolinol were carried out by injecting 40-μl aliquots of the samples onto the nucleodex-β-OH column. The column was eluted isocratically at a flow-rate of 0.5 ml/min. Detection was performed electrochemically with a high sensitivity analytical cell set at a potential of 0.4 V. Plasma concentrations of dopamine, *R*- and *S*-salsolinol were calculated from area integrations using a working standard solution as reference. The elution order of the enantiomers of salsolinol was determined using nearly pure *S*-salsolinol-HBr as reference.

Resolution between the peaks of the two enantiomers is 3.38 (calculated by the equation $R_S = 2(t_{R2} - t_{R1}) \cdot [1.699(W_1 + W_2)]$, where t_{R2} and t_{R1} are the retention times and W_1 and W_2 are the widths at half height of the peaks.

The column efficiency was at least 7000 (calculated by the equation $N = 5.54(t_R/W)^2$, where t_R is the retention time of the peak and W is the peak width at half height.

The reproducibility of the HPLC system is shown in Table 1. The working standard was injected five times on 12 consecutive days. The resulting peaks were measured in mm peak height and the means as well as the variance were calculated (C.V.: coefficient of variation). The table shows a low standard deviation and the C.V. was within ± 5% for all three amines. The response factor of the two enantiomers, *S*-SAL/*R*-SAL, was calculated from the peak height, and gave a factor of 1.039 ± 0.022 (mean ± S.D.). The coefficient of variation was 0.014.

The precision of the system was tested by injecting different amounts of racemic salsolinol (0.02–1.04 ng) and calculating the response factor *S*-SAL/*R*-SAL from the equation:

$$\frac{\text{peakheight (mm)}}{\text{amount injected (ng)}}$$

which gave a factor of 1.095 ± 0.04 (mean ± S.D.) and a coefficient of variation of 0.039.

The relative retentions for dopamine, *S*-sal-

Table 1
Precision of the system reproducibility working standard (inter-day)

Compound	Peak height (mean ± S.D.) (mm)	C.V. (%)
Dopamine	105.04 ± 7.85	5.07
S-Salsolinol	61.28 ± 1.96	1.26
R-Salsolinol	59.02 ± 2.04	1.32

solinol and *R*-salsolinol [peak height (mm)/ amount injected (ng)] were 148.4 ± 11.1, 70.4 ± 2.2 and 74.9 ± 2.5, respectively. The observed enantioselectivity (α) for *R*-salsolinol and *S*-salsolinol was unity.

3. Results

3.1. Range of applicability of the procedure

A typical chromatogram of the authentic standard solution is shown in Fig. 2.

Blank samples showed no peaks interfering with the dopamine and *R*- and *S*-salsolinol peaks and only a solvent peak was recorded. The HPLC procedure affords excellent separation of the three catecholamines. Assuming a signal-to-noise ratio of at least three, the detection limits of the assay correspond to plasma concentrations of 0.02 ng/ml for dopamine, *R*- and *S*-salsolinol respectively. Fig. 3 shows two chromatograms from a native plasma sample and from the same plasma sample with standards added prior to processing the sample.

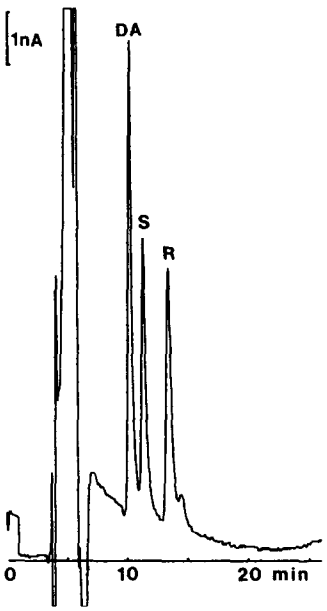

Fig. 2. HPLC elution profiles of the authentic standard solution peaks: DA = dopamine (t_R = 10.6), S = *S*-SAL (t_R = 11.8), R = *R*-SAL (t_R = 14.1).

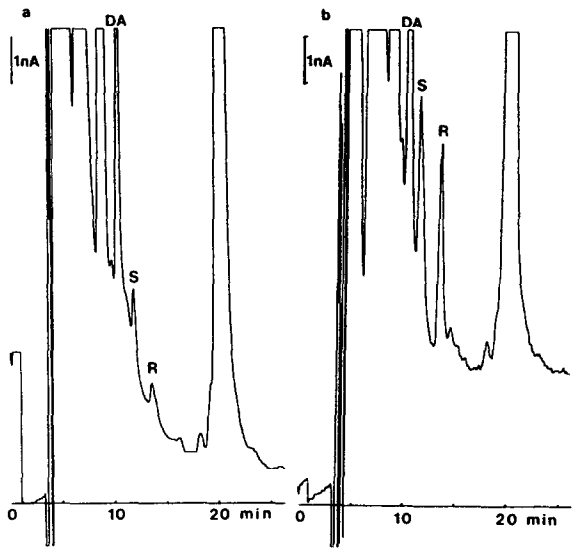

Fig. 3. HPLC elution profiles of (a) a native plasma sample (from an alcoholic patient), and (b) the same plasma sample spiked with dopamine and racemic SAL before processing the sample. Peaks: DA = dopamine, S = *S*-SAL, R = *R*-SAL.

3.2. Linearity

Concentrations and peak-height ratios were linearly related over the measured ranges from 0.02 to 0.44 ng injected (gain 9500) and from 0.16 to 0.8 ng injected (gain 4000). The correlation coefficients were 0.997, 0.990 and 0.990 for dopamine, *S*-SAL and *R*-SAL, respectively.

3.3. Precision and recovery

In order to investigate the within-day and the between-day precision of the method, several aliquots of one and the same acidified pooled plasma sample spiked with dopamine, *R*- and *S*-salsolinol were stored at pH 4 and −80°C. These samples were analyzed for dopamine, *R*- and *S*-salsolinol on consecutive days. The results are given in Table 2.

The table shows the recoveries of the dopamine, and *R*- and *S*-salsolinol added to the plasma. The coefficients of variation (C.V.) of the within-day assay and the between-day assay

Table 2
Precision of the assay of plasma dopamine and *S*- and *R*-salsolinol

	Plasma[a] (ng/ml)			Recovery[a] (%)		
	DA	*S*-SAL	*R*-SAL	DA	*S*-SAL	*R*-SAL
Within-day precision (n = 8)						
Mean	9.42	3.87	3.31	100.51	84.67	90.16
S.D.	0.39	0.24	0.12	10.96	7.10	5.71
C.V.%	0.41	0.25	0.11	–	–	–
Between-day precision (n = 6)						
Mean	8.10	3.61	3.00	104.32	96.81	88.02
S.D.	0.39	0.29	0.24	10.63	7.65	7.14
C.V.%	0.41	0.30	0.26	–	–	–

[a] The endogeneous concentrations were increased by 3.6 ng/ml dopamine, 3.75 ng/ml *S*-SAL and 3.42 ng/ml *R*-SAL.

were both small. The response factor for both enantiomers, *S*-SAL/*R*-SAL, for the within-day assay was 1.032 ± 0.089, with a C.V. of 0.08, and for the between-day assay 1.17 ± 0.084, with a C.V. of 0.08.

3.4. Capacity of the PBS and PBA cartridges

To determine the adsorption capacity of the Bond Elut cartridges PSA and PBA to adsorb the amines from plasma, aliquots of a pooled plasma sample were spiked with different amounts of dopamine, *R*- and *S*-salsolinol in the range between 0 and 40 ng/ml and 0–20 ng/ml, respectively. The capacity of the cartridges was sufficient to adsorb the amines in concentrations up to at least six times (for dopamine) and up to 16 times (for *R*- and *S*-salsolinol) the concentrations found in plasma of normal individuals.

3.5. Interference by structurally related compounds

The cartridges elute all amines from plasma that are basic compounds with both a vicinal hydroxyl configuration and a primary or secondary amino group. We could not find any co-eluting compounds, such as adrenaline, noradrenaline or serotonin, interfering with the peaks of interest by utilizing the assay as described in this paper.

3.6. Free and conjugated amines

The ratio between free and conjugated dopamine or free and conjugated salsolinol in human plasma as determined by using the assay with or without the hydrolysis step showed that 96.4–98.6% of dopamine and 90–100% of salsolinol is present in a conjugated form in the plasma.

4. Discussion

In the analysis of tetrahydroisoquinolines with HPLC, urine samples were usually used by most investigators. We assumed that determination of salsolinol in plasma would provide us with more accurate information concerning the function of salsolinol in normal subjects and in pathological cases. Urine must be collected, usually over a 24-h period, a procedure which can not be adequately controlled. Dietary influences can not be adequately controlled and kidney dysfunctions may affect the results. Therefore, the aim of the present study was to develop a HPLC method for determining the amount of salsolinol and dopamine in plasma.

Recently, microparticulate (e.g. 5 μm particle size) chemically bonded ion-exchange materials have become available which overcome some of the earlier problems with respect to reproducibil-

ity and stability [13]. The use of a primary/secondary amine resin as a precolumn for the phenylboronic acid treatment serves two purposes: it removes some potentially interfering amines that would be retained by the cyclodextrin analytical column and would be detected if electrochemically active at 0.4 V and it also increases the pH of the sample by adsorbing hydrogen ions. Samples need only to be adjusted to pH 5.0 before passage through the PSA column, which will change the pH to 8.5. The samples treated in this way are immediately transferred to the PBA column for adsorption. Because the amines are more stable at pH 5.0, there is less oxidative loss of the amines compared with the case where the sample is subjected to prolonged exposure to pH 8.5.

Under the conditions described, dopamine and SAL were retained quantitatively by the cartridges and were almost quantitatively eluted with phenylboronic acid. This step, combined with the optimized hydrolysis procedure, gave excellent recoveries.

Our attempts to hydrolyse the plasma samples with β-glucuronidase–arylsulphatase (using enzymes from 4 different manufactures) resulted in too many interfering HPLC peaks. Although this procedure is usually used for work-up of urine samples we did not find it suitable for processing plasma samples.

Several arguments support the assumption that the compounds eluting after dopamine in the HPLC chromatograms were actually SAL. We used two other different eluents (trifluoracetic acid eluents and citric acid eluents) to prove that the detected peaks of dopamine, *S*-SAL and *R*-SAL had the same elution times as the standards. We also used ion-pair reversed-phase chromatography with an ODS-II column (5 μm). This demonstrated that the unseparated *R*-SAL and *S*-SAL in the plasma sample, as well as dopamine, eluted isographically with the standards.

Comparison of the methods used for the determination of the conjugated amines shows that, despite their different absolute values, the relative proportions of the free and conjugated dopamine and probably also of the free and conjugated *R*- and *S*-salsolinol are rather constant (97.3% of dopamine and more than 80% of *R*- and *S*-salsolinol are conjugated) and that these proportions did not change, regardless whether acid or sulphatase hydrolysis is used [14]. The variability is rather due to the method of amine determination than to the mode of hydrolysis of the conjugated amines. Reliable calibration curves were obtained for the quantification and determination of the enantiomeric composition of salsolinol and for dopamine in human plasma.

The described method offers some advantages over methods using a derivatization reagent where the best agents are difficult to obtain. The chiral medium used gives excellent separation of the free forms of the two enantiomers of salsolinol.

Our values for *R*- and *S*-salsolinol (mean: 0.397; range: 0–0.95) and for dopamine (mean: 3.70; range: 1.11–6.68) ($n = 15$) are not quite comparable with the reference values for human plasma reported by Faraj et al. [2] who used a radioimmunoassay [mean: 0.093, range: 0–0.232 ng/ml for racemic salsolinol; mean: 2.67, range: 0.86–6.27 ng/ml for dopamine ($n = 36$)]: the dopamine as well as *R*- and *S*-salsolinol values are higher. Odink et al. [15] found dopamine values between 3.4 and 5.1 ng/ml plasma, Ratge et al. [16] found 4.83 ng/ml and Dousa et al. [17] found 5.05–7.39 ng/ml plasma in healthy subjects which is in the same order of magnitude as the dopamine concentrations found in the present study.

The unimolecular acid-catalyzed hydrolysis of dopamine and salsolinol-sulphate proved to be simple and fast, with very good recovery of the amines spiked into plasma. Sulphatase-catalyzed hydrolysis of aminesulphate was also quantitative for urine samples, but less than 20% efficient with plasma samples [18]. We conclude that the rather specific and easy-to-handle procedure with boronic acid cartridges used for the isolation of plasma free and conjugated dopamine, *R*- and *S*-salsolinol, together with the high resolving power of HPLC operated in the chiral mode and the high sensitivity of the electrochemical detector provide a fast and reliable method for the

determination of the three plasma catecholamines.

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