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DETERMINATION OF THE ENANTIOMERIC COMPOSITION OF SALSOLINOL IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

The enantiomers of salsolinol were completely separated as diastereoisomeric derivatives, after reaction with *S*-1-(1-naphthyl)ethyl isothiocyanate, by reversed-phase high-performance liquid chromatography and quantified by electrochemical detection Good calibration curves were obtained for the quantification and determination of the enantiomeric composition of salsolinol in human urine The sensitivity and specificity to the assay also permit the determination of the enantiomeric composition of salsolinol in food such as dried bananas and chocolate

INTRODUCTION

Salsolinol (1-methyl-1,2,3,4-tetrahydro-6,7 isoquinolinediol) (Sal) is an endogenous tetrahydroisoquinoline alkaloid which has been detected in human urine, cerebrospinal fluid and brain tissue [1,2] Variable concentrations

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of urinary Sal were detected in physiological and pathological conditions such as Parkinson's disease [3] and alcoholism [4] It has been suggested that modification of Sal concentrations in human tissues and fluids could correlate with some pathophysiological states, such as alcoholism and Parkinson's and Huntington's diseases [5]

Sal concentrations in biological samples were successfully measured by gas chromatography (GC) with electron-capture detection [6], gas chromatography–mass spectrometry (GC–MS) [3,7] and ion-pair liquid chromatography with electrochemical detection (ED) [8,9]

Sal possesses an asymmetric centre at C-1 and exists as *S* and *R* enantiomers (Fig 1) In all the above-mentioned papers, concentrations in total Sal ($R+S$) were measured As the two enantiomers of Sal do not display identical biological activities [10] it appeared that a new method for determining the enantiomeric composition of endogenous Sal in biological samples would be very useful

A method distinguishing between *R* and *S* Sal in human urine was first reported by using GC and nitrogen–phosphorous detection after derivatization with diazomethane and then with N-trifluoroacetyl-L-prolyl chloride [11]. However, that method did not allow a suitable quantification of the *S* enantiomer, although it did establish that the *R* enantiomer predominates in human urine

The same authors also reported a high-performance liquid chromatographic (HPLC) assay by which the relative proportion of the two Sal enantiomers was quantified in human urine and in some foods and beverages [12] In this method, a Resolvosil®-BSA chiral column and ED were used without derivatization, and a limit of detection of ca. 50 pg/ml urine was found for both enantiomers However, repeated attempts to reproduce this separation in our laboratory by using a similar column proved unsuccessful Then a new HPLC method was assayed [13] It was based on derivatization of Sal with N-trifluoroacetyl-L-prolyl chloride, selective hydrolysis of the phenolic esters and detection of the resulting amide diastereoisomers by ED after HPLC separation This method, although sufficiently sensitive, precise and accurate, had two main drawbacks· the time required for the formation of diastereoisomers was relatively long (ca 2 h) and the chromatographic separation of the two compounds was not fully satisfactory. As our efforts to achieve a better separation by changing either the mobile or the stationary phase failed, we decided to

(R)SALSOLINOL (S)SALSOLINOL

Fig 1 Structures of the Sal enantiomers

change the derivatizing agent The new optically active reagent to be selected was required to react selectively with the amino group of the Sal and to give stable diastereoisomers easily separable by HPLC Gal et al [14] described a chiral reagent *S*-1-(1-naphtyl)-ethyl isothiocyanate (S-NEIT) that seemed to fulfil both these demands This paper reports the setting up of a new HPLC method for the quantification of the two enantiomers of Sal as diastereoisomers derived from S-NEIT

EXPERIMENTAL

Compounds

(±)-Salsolinol hydrobromide was purchased from Roth (Karlsruhe, F R G) and *S*- and *R*-salsolinol were prepared according to Teitel et al [15] S-NEIT was synthesized by Gal and Desai (University of Colorado, Denver, CO, U S A) Phenyl boronic acid (PBA) cartridges were from Analytichem International (Harbor City, CA, U S.A), all other chemicals and solvents were analytical grade from Farmitalia–C Erba (Milan, Italy)

Apparatus

A Milton Roy Constametric 3000 pump equipped with a Rheodyne 7125 injection system and a 50-μl sample loop was used The detection system consisted of an ESA Model 5100A electrochemical detector equipped with a Model 5011 graphite cell (5-μl volume) and connected to a Spectra-Physics Model SP 4290 recorder–integrator A Millipore/Waters Novapak-C_{18} column (15 cm × 3 9 mm I.D , particle size 4 μm) was used The mobile phase was a mixture of methanol and 50 m*M* phosphate buffer, pH 3 0 (55 45, v/v), and the flow-rate was 1 0 ml/min

Extraction

PBA cartridges were used for a selective liquid–solid extraction of Sal The cartridge was conditioned before the loading of the sample by washing sequentially with 1 ml of methanol, 1 ml of water, 1 ml of 0 1 *M* hydrochloric acid, 1 ml of 0 3% ammonium hydroxide and finally 1 ml of 0.01 *M* (pH 8 5) ammonium sulphate buffer (twice) Then the sample buffered at pH 8.5 was loaded on the top, and passed through the cartridge by gentle vacuum aspiration After three washings with water (1 ml) and one with methanol (1 ml), the absorbed Sal was eluted from the cartridge with 1 *M* acetic acid in methanol (0 5 ml, twice) The eluate was evaporated to dryness at room temperature under vacuum and then derivatized

Derivatization

Triethylamine [100 μl of a 0.3% (v/v) solution in acetonitrile] was added to the extraction residue, followed by an acetonitrile solution of S-NEIT (100

μl of a 0 1 mg/ml solution) After stirring, the reaction mixture was heated for 30 min at 40°C under nitrogen Ethanolamine [100 μl of a 0 2% (w/v) solution in acetonitrile] was then added, and the mixture was kept for 10 min at 40°C in order to eliminate the excess S-NEIT Then the solution was evaporated at room temperature under vacuum to dryness, and the residue was dissolved in 140 μl of a 50 m*M* orthophosphoric acid–methanol solution (1 1, v/v) Aliquots (50 μl) of this solution were injected onto the column

Determination of the enantiomeric composition

Analyses of standard solutions containing known amounts of racemic Sal were carried out to determine the response factors of the two enantiomers Standard curves of both compounds were obtained in the range 2 5–150 ng The precision of the method was determined by repeated analyses of known amounts of racemic Sal in the whole range of the standard curve points The accuracy was determined by processing samples of unknown racemic Sal amounts and expressed as the percentage ratio of found/added amount All the chromatograms obtained were evaluated by peak-height measurement and external calibration The mean recovery was evaluated by measuring the peak-height ratio of processed Sal solutions with respect to the peak height of the same solutions subjected only to the derivatization reaction

Chromatographic system suitability test

System reproducibility Before use the chromatographic column was equilibrated with the mobile phase until four consecutive injections of a sample containing ca 5 ng of both standard Sal diastereoisomers produced responses with a coefficient of variation (C V) within $\pm 5\%$

Column efficiency It was evaluated as the number of theoretical plates of the column calculated by the equation $N = 5\,54\ (t_R/W)^2$, where t_R is the retention time of the peak of a derivatized enantiomer and W is the peak width at half-height The value of N must be at least 4000

Peak resolution The resolution factor between the peaks of the two diastereoisomers was calculated by the equation $R_s = 2(t_{R2} - t_{R1})\,[1.699(W_1 + W_2)]^{-1}$, 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 of the two diastereoisomers The value of R_s must be more than 1 0

RESULTS

The diastereoisomeric mixture obtained by processing racemic Sal was injected onto the HPLC column and analysed with different mobile phases, owing to the poor stability of the catechol group in basic solution, only the acidic range of pH values was explored to define the best composition of the mobile phase

A fast and reliable separation of the two compounds was obtained by using a Millipore/Waters Novapak C_{18} column eluted isocratically with methanol–50 m*M* pH 3.0 phosphate buffer (55 : 45, v/v). The peaks corresponding to the two compounds were well separated ($R_s = 1.18$) and their relative peak-height ratio was 1.1 (see Fig. 2).

The analyses of each single derivatized enantiomer revealed that the *R* isomer of Sal was eluted before the *S* isomer.

The characteristics of the detector used made it possible to operate in the redox mode (first electrode voltage +500 mV, second electrode −200 mV), thus considerably diminishing the number of interfering peaks in the chromatogram. Norepinephrine and dopamine standard solution processed as de-

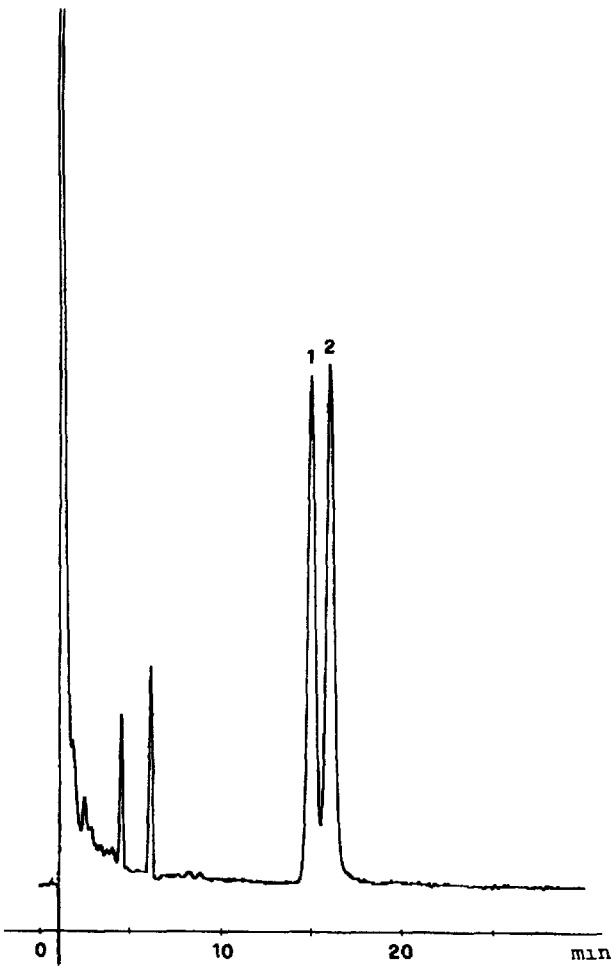

Fig. 2. Typical chromatogram obtained by processing a standard solution of racemic Sal (50 ng) as described in the text. Peaks: 1 = *R*-Sal ($t_R = 15.0$ min), 2 = *S*-Sal ($t_R = 16.0$ min).

TABLE I

PRECISION OF THE METHOD (INTRA-DAY ASSAY)

Amount injected ($n=3$) (ng)	First day		Second day		Third day	
	RF_R[a] (mean ± S D)	RF_S[a] (mean ± S D)	RF_R (mean ± S D)	RF_S (mean ± S D)	RF_R (mean ± S D)	RF_S (mean ± S D)
49 80	892 33 ± 16 02	895 67 ± 16 45	1049 59 ± 38 89	1103 93 ± 25 75	881 58 ± 19 47	842 24 ± 18 61
35 63	974 59 ± 92 58	973 83 ± 92 44	976 43 ± 59 94	982 79 ± 68 54	948 15 ± 99 16	918 40 ± 85 88
21 38	953 69 ± 57 53	943 94 ± 85 94	1144 65 ± 61 56	1089 34 ± 98 17	966 18 ± 63 53	860 68 ± 43 74
13 58	1038 44 ± 122 45	993 19 ± 92 99	983 51 ± 47 14	774 69 ± 84 01	935 88 ± 122 77	806 93 ± 179 37
3 69	878 23 ± 103 37	875 33 ± 84 29	1194 29 ± 40 67	964 82 ± 61 27	1084 21 ± 67 28	1012 81 ± 66 04
0 922	790 57 ± 92 78	806 46 ± 74 73	1194 55 ± 22 69	1160 92 ± 85 34	1098 51 ± 46 57	1105 28 ± 99 11
	921 31 ± 109 82	914 74 ± 93 14	1098 00 ± 100 76	999 20 ± 132 40	980 36 ± 99 49	924 40 ± 134 11
C V (%)	11 92	10 18	9 17	13 15	10 15	14 55

[a] $RF_{R/S}$ are the response factors of the two enantiomers, calculated from the following equation $\frac{\text{peak height (digit mV/s)}}{\text{amount injected (ng)}}$

TABLE II

PRECISION OF THE METHOD (INTER-DAY ASSAY) ($n=54$)

RF_R (mean ± S D)	C V (%)	RF_S (mean ± S D)	C V (%)
921 31 ± 109 82	11 92	914 74 ± 193 14	10 18
1098 00 ± 100 76	9 17	999 20 ± 132 50	13 26
980 36 ± 99 49	10 15	924 40 ± 134 30	14 55
1001 74 ± 122 52	12 23	946 11 ± 125 17	13 23

TABLE III

ACCURACY OF THE METHOD

Amount added (ng)	Amount found (mean, $n=3$)	$\frac{\text{Found}}{\text{Added}}$ ± S D (%)	C V (%)
R-Sal			
165 5	164 2	99 22 ± 9 10	9 17
25 76	26 73	103 77 ± 4 96	4 78
2 79	2 94	105 62 ± 15 39	14 57
Overall average ($n=9$)		102 87 ± 9 70	9 43
S-Sal			
165 5	163 06	98 53 ± 9 35	9 49
25 76	26 86	104 27 ± 6 25	5 99
2 79	2 98	106 79 ± 19 54	18 30
Overall average ($n=9$)		103 20 ± 11 86	11 49

scribed gave single peaks, which did not interfere with those of interest (5 92 min and 8 55 min, respectively) Salsoline, the methylated derivative of salsolinol, was not retained by the cartridge, showing that the overall specificity of the assay was sufficient to guarantee the selective determination of the two Sal enantiomers The linearity was evaluated for each Sal enantiomer from six separated calibration curves carried out in the whole range of measurement on different days Intercept values, when submitted to the statistical SAS test, were not significantly different from zero ($p>0$ 05) The mean calculated parameters for the *R* enantiomer were correlation coefficient $r=0$ 9952 (C V = 0 31%), slope = 945 33 (C V = 9 13%), intercept = 680.11 (C V = 85 27%) Those for the *S* enantiomer were correlation coefficient $r=0$ 9943 (C V = 0 375%), slope = 947 23 (C V = 12 14%), intercept = − 78 41 (C.V = 1319 6%) The C V. of the (intra- and inter-day) pre-

cision of the method was found to be 12 23 and 13.23% for the *R* and *S* enantiomer, respectively (Tables I and II). The accuracy over the whole concentration range of interest, calculated with samples analysed on different days and reported as the mean percentage ratio of the found/added amounts ± C V., was 102 87 ± 9.43 and 103.20 ± 11 49 for the *R* and *S* enantiomer, respectively (Table II) The minimum detectable amount was assumed to be the lowest validated point of the standard curve, i.e. 2 5 ng for each enantiomer analysed

The total recovery was calculated for each enantiomer at three concentrations (3, 15 and 100 ng/ml) from five series of replicated analyses, the mean values obtained were 42.52% (S.D. = 5.78–3.62) and 40 55% (S D. = 5 59–3.73) for the *R*- and *S*-enantiomer, respectively.

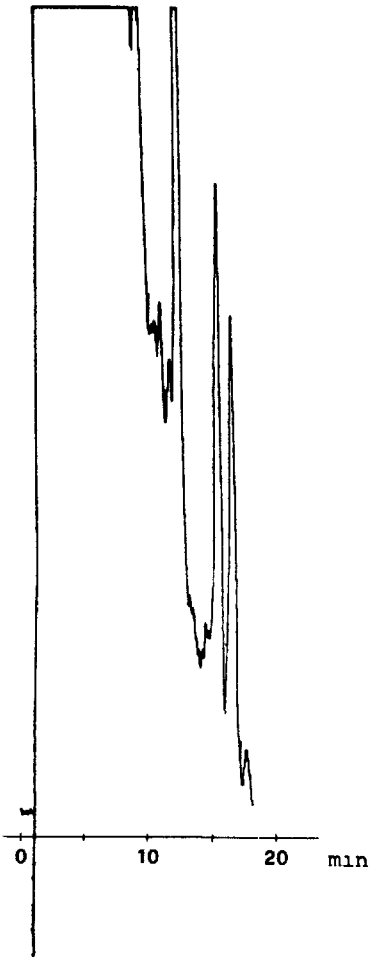

Fig 3 Determination of the two Sal enantiomers as S-NEIT derivatives Chromatogram obtained from human urine processed and analysed as described in the text

DISCUSSION

The described method offers several advantages over the one previously described [13] The derivatization reagent reacts rapidly and selectively with the amino group of both Sal enantiomers giving a stable pair of diastereoisomers whose physicochemical properties make them easily separable by HPLC Moreover, although the sensitivity was not improved, better results in terms of accuracy and reproducibility were obtained by using this assay due to the excellent resolution of the two diastereoisomers

The method enabled us to measure the enantiomeric composition of Sal in samples of human urine from healthy volunteers and patients A typical chromatogram of a urine sample obtained from a healthy volunteer who had ingested dried banana is shown in Fig 3

The method was also used to quantify the enantiomeric composition of foods

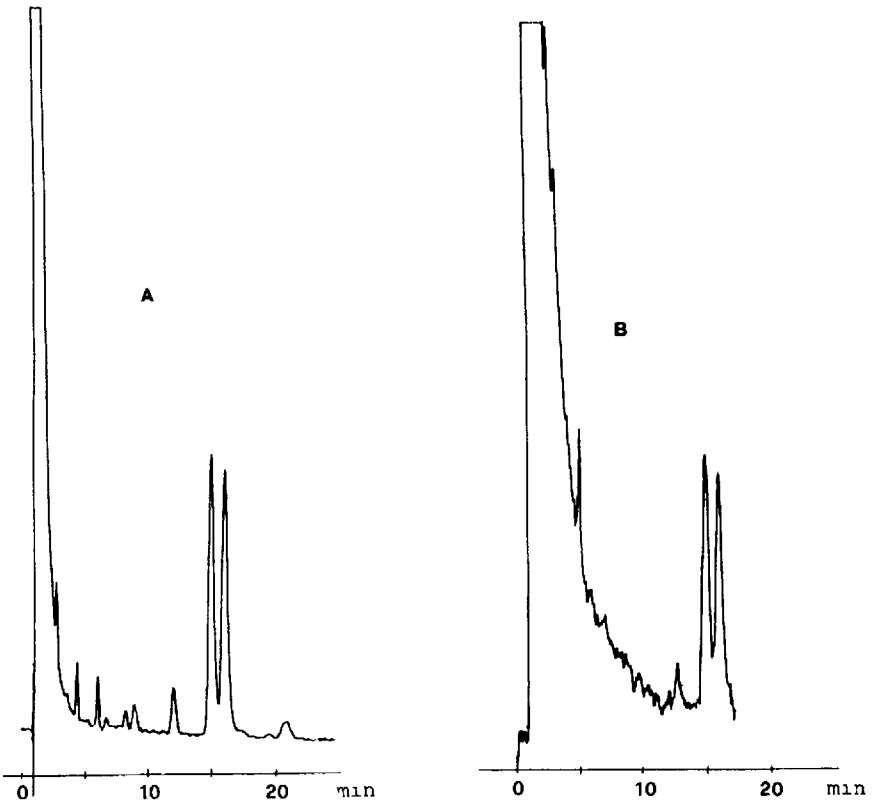

Fig 4 Chromatograms obtained from samples of dried banana (A) and chocolate (B), showing the lack of interferences in the chromatographic determination of the two Sal enantiomers as S-NEIT derivatives

rich in Sal, such as dried bananas and chocolate no interferences from the matrix were observed (see Fig 4)

Using this method, the intake of dried banana was recently shown to influence the urinary excretion of Sal enantiomers [16]

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