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Note

Rapid high-performance thin-layer chromatography of salicylic acid, salicylamide, ethoxybenzamide and paracetamol in saliva

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The determination of salivary drug concentrations is gaining increasing interest for pharmacokinetic studies. For a number of drugs the saliva/plasma ratio is well established. The mechanism as well as determining factors of salivary excretion of drugs have been discussed in detail [1—9]. As salivary drug concentrations are usually lower than plasma concentrations the analytical procedure has to be especially sensitive and should be suitable for routine work.

A large number of papers report the determination of weak analgesics generally with the emphasis on separating the parent drug from its metabolites. Chromatographic methods include thin-layer chromatography (TLC) [10,11], gas chromatography [8], and high-performance liquid chromatography [12—14]. TLC has the advantage of simplicity and allows the simultaneous determination of several samples in one run. With the recent advent of high-performance TLC (HPTLC) a higher precision is attainable than with classical TLC—densitometry and analysis times are considerably shortened [15,16].

TABLE I

COMPARISON OF METHODS FOR ANALYSIS OF SALICYLIC ACID

Reference	Analytical method	Sample	Sensitivity	Reproducibility (%)
10	TLC—densitometry	Tablets	75 µg/spot	1.31
11	TLC—densitometry	Serum	111 µg/spot	4.50
13	HPLC	Plasma	200 µg/ml	3.82
This paper	HPTLC	Saliva	1 µg/ml	13.10
			= 40 ng/spot	5.30

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Table I provides a comparison of recent work done on salicylic acid with our results. The HPTLC method proves to be more sensitive; however, the error increases proportionally at lower concentrations, especially in the nanogram range. Consequently, the final judgement should only be made on the basis of work done with saliva which is discussed later in the text.

EXPERIMENTAL

Apparatus and materials

The reference substances — salicylic acid, salicylamide, ethoxybenzamide, paracetamol — all analytical grade, were purchased from Merck (Darmstadt, G.F.R.). All the reagents (chloroform, acetone, ammonium sulfate, formic acid and dichloroethane) were of analytical grade (Merck).

Extraction procedure

The extraction of salicylic acid, salicylamide, ethoxybenzamide, and paracetamol was performed on a Vortex whirlmixer as follows. Two grams of ammonium sulfate and 1 drop of concentrated sulfuric acid were added to 2 g of saliva and mixed for 30 sec. Then 2 ml of chloroform were added; the tube was sealed by a glass stopper and shaken vigorously for 15 min. After centrifugation the aqueous layer was removed and the chloroform was pipetted into a 3-ml conical flask. The tube was washed twice with 0.5 ml of acetone and then added to the chloroform phase. The solvents were evaporated under a stream of nitrogen. A 0.1-ml volume of acetone was pipetted (enzyme pipette) into the flask, dissolving the residue with the aid of the mixer.

Chromatography

Chromatography was performed on 10 cm × 20 cm HPTLC plates coated with silica gel 60 (Merck). Standard and test solutions (2-μl volumes) were applied to the HPTLC plate using a Mikroliter Applicator (Merck) and 2-μl glass capillaries (Merck).

A stock solution in acetone and three dilutions in acetone were prepared weekly and kept cool and in the dark. The stock solution was 1 mg/ml for paracetamol and the dilutions 200, 100 and 25 μg/ml. The stock solutions for salicylic acid, salicylamide and ethoxybenzamide were each 100 μg/ml and the dilutions for each were 20, 10 and 2.5 μg/ml. All dilutions proved to be stable for at least one week. They were used for the standard curve and had to be chromatographed on each plate.

For each plate 19 spots were applied 1 cm apart in the sequence standard—test solution—standard; there were 3 spots for each standard concentration, leaving 10 spots for test solutions. The spot diameter was less than 2 mm. The starting point was kept constant at 1 cm from the edge of the HPTLC plate by means of the Mikroliter Applicator.

The chromatography solvent system consisted of concentrated formic acid—dichloroethane (1:10, v/v) and was suitable for all compounds. The inside of a tank for ascending chromatography (Camag, Muttenz, Switzerland) was lined with filter paper to accelerate saturation which was reached after 45 min.

The solvent system could be used for three plates on the same day at room temperature.

The plates were developed for 9 cm, which corresponds to 20 min, and were allowed to dry in the air for 15 min. The chromatograms were scanned in situ with a PMQ 3 densitometer (Zeiss, Oberkochen, G.F.R.). The spectrophotometer parameters were: slit length 7 mm, slit width 0.7 mm, scanning speed 120 mm/min. The scans were recorded on a Metrawatt RE 647; integration of spot areas (by product of peak height and width at one half the peak height) was performed using a Spectra-Physics Minigrator (Spectra-Physics, Santa Clara, CA, U.S.A.). Calculation of the concentration in each saliva sample was made from the standard curve obtained for each plate. The standard curves for all of the substances were linear, the correlation coefficient being at least $r = 0.998$.

Salicylic acid, salicylamide and ethoxybenzamide were measured by their fluorescence: excitation wavelength 314 nm, emission wavelength 390 nm, filter. Paracetamol was measured by its light absorption at 247 nm, using remission. The R_F values obtained were: paracetamol, 0.13; salicylamide, 0.43; ethoxybenzamide, 0.56; salicylic acid, 0.62.

Blank saliva from different subjects was chromatographed on the same plate with each substance. There was only one spot detectable by UV at 247 nm for an unknown salivary compound. The R_F value of the tiny peak was 0.06; the separation from paracetamol was sharp. While measuring fluorescence no detectable spot from blank saliva over the whole plate could be observed under our photometric conditions.

RESULTS AND DISCUSSIONS

Accuracy

The concentration range for the paracetamol standard curve was 400, 200, 50 ng/spot, 50 ng being the limit of practical sensitivity. The concentration range for salicylic acid, salicylamide, ethoxybenzamide standard curve was 40, 20, 5 ng/spot, 5 ng being the limit of practical sensitivity for salicylic acid, and 2.5 ng the limit of practical sensitivity for salicylamide and ethoxybenzamide.

The accuracy of the scanning measurement was determined as instrument error — one spot was measured three times — and as total instrument error — one standard concentration was measured on six different spots, three times. Table II presents the accuracy data for all standard curves, expressed as the relative standard deviation.

Recovery

Preliminary experiments had indicated the concentration ranges which were to be expected after intake of a particular drug. We prepared saliva samples with blank saliva obtained from different subjects, and added 0.1 ml of acetone containing the drug concentration of interest. To assess the recovery in routine work we decided to prepare and extract two samples each day over a period of six successive days. From twelve saliva samples we calculated our recovery data in the following ranges: paracetamol, 2.5 and 5 μg/

TABLE II

COMPARISON OF THE SCANNING MEASUREMENT TO THE TOTAL INSTRUMENT ERROR

	Concentration (ng/spot)	Instrument error (rel. S.D. %)	Total instrument error (rel. S.D. %)
Paracetamol	400	<1.0	2.36
	200	<1.6	2.07
	50	<6.0	6.3
Salicylic acid	40	<1.2	5.3
	20	<3.0	4.0
	5	<5.0	15.0
Salicylamide	40	<0.7	1.0
	20	<1.0	1.35
	5	<3.0	6.8
Ethoxybenzamide	40	<0.6	2.74
	20	<1.2	4.3
	5	<3.0	4.42

TABLE III

MEAN AND STANDARD DEVIATION OF THE EXTRACTION PROCEDURE FROM SALIVA

Substance	Concentration (μg/ml)	Recovery (%) (n = 12)
Paracetamol	5	$\bar{X}$ = 96.1 S.D. = 11.94 rel. S.D. = 12.4
	2.5	$\bar{X}$ = 84.0 S.D. = 20.88 rel. S.D. = 24.9
Salicylic acid	1	$\bar{X}$ = 74.6 S.D. = 9.8 rel. S.D. = 13.1
	0.5	$\bar{X}$ = 87.0 S.D. = 10.5 rel. S.D. = 12.0
	0.25	$\bar{X}$ = 77.5 S.D. = 15.5 rel. S.D. = 20.0
Salicylamide	0.25	$\bar{X}$ = 78.2 S.D. = 13.6 rel. S.D. = 17.4
Ethoxybenzamide	0.25	$\bar{X}$ = 80.15 S.D. = 6.4 rel. S.D. = 8.0

ml; salicylic acid, 0.25, 0.5, 1 μg/ml; ethoxybenzamide, 0.25 μg/ml; salicylamide, 0.25 μg/ml. The data obtained are presented in Table III. The extraction ranges given here correspond to the average concentrations which were obtained in preliminary experiments after therapeutic doses.

Comparison with data from earlier work done with saliva

While comparing our results with literature data we found that paracetamol had been extracted from saliva in the range 20—5 μg/ml but neither recovery data nor standard deviations were given [2,17].

To our knowledge ethoxybenzamide has not before been extracted from saliva.

Salicylamide has been studied in the range 25—1 μg/ml with an extraction yield of 83%, but no standard deviation was given [8]. Salicylic acid was analyzed in the range 1.2—0.08 μg/ml with standard deviations of 2—25% [1], which is in agreement with our results.

Pohto [7] determined salicylic acid in the range 1.2—0.5 μg/ml by UV measurement at 277 and 300 nm, with better reproducibility compared to our HPTLC method. However, his analytical procedure would be inconvenient for studying salivary drug concentrations after administration of tablets containing additional different drugs.

In conclusion, we believe that the method described here is convenient for bioavailability studies and in routine therapeutic monitoring.

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