SEPARATION AND IDENTIFICATION OF CAFFEINE, ANTIPYRINE AND PHENACETIN FROM HUMAN TISSUE

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Upon extraction of acidified tissue homogenates with organic solvents, neutral and acidic compounds of various kinds are obtained. Among these substances barbiturates are of special toxicological interest. In the course of barbiturate analyses performed in this laboratory, it was found that three other compounds, caffeine, antipyrine (phenazone), and phenacetin (acetophenetidine), which are extracted together with the barbiturates, can be easily separated, identified and assayed at the same time as the barbiturates with the help of paper chromatography.

Separation of these three compounds from each other has been achieved by SJÖSTRÖM[1], using ion exchange columns; similar methods for the separation of one or two of these substances from each other and from contaminants have been reported. Our standard procedure is as follows: The tissue to be analyzed (as a rule 30 g portions of either liver, kidney, blood or urine) is acidified to pH 3 and extracted with chloroform, both before and after acid hydrolysis. After evaporation to dryness, the residue is freed from fat by treatment with very dilute warm acid and filtration in the cold. The filtrate is extracted with chloroform. Strongly acidic compounds are removed from the chloroform extract with phosphate buffer of pH 7.2. Caffeine, antipyrine, and phenacetin are almost quantitatively transferred to the final chloroform extract. The distribution coefficients for the three compounds are listed in Table I. The "washed" chloroform extract is now analyzed by descending chromatography on Whatman No. 1 paper (25 × 50 cm) that has been treated with 0.05 *M* Na_2CO_3 solution. Standard solutions of the 10 most common barbituric acid derivatives are used as controls on the same sheet. Water-saturated chloroform, diethyl ether or di-*n*-butyl ether are used as the mobile phase. The running time is about 1.5 hours for

TABLE I

DISTRIBUTION COEFFICIENTS BETWEEN CHLOROFORM AND WATER*

Chloroform	*Caffeine*	*Antipyrine*	*Phenacetin*
0.1 *N* HCl	16.5	5.5	28.4
Phosphate buffer pH 7	24.6	17.5	> 100
0.5 *N* NH_4OH	16.5	26.8	32.4

* Ratio of concentrations of the solute in chloroform and in the aqueous phase, using equal volumes of each solvent.

ethyl ether, about 2 hours for chloroform, and 5 hours for *n*-butyl ether. In the last case, the solvent front is allowed to leave the paper.

Paper chromatography of barbiturates is usually carried out with chloroform as the mobile phase[2], though butanol or amyl alcohol have also been employed. Table II shows that with chloroform as a solvent the three compounds discussed here all move close to the solvent front, and thus are apt to be lost in tissue impurities. But with ether as the moving phase, separation is excellent. However, these compounds can be confused with certain barbiturates: caffeine and phenobarbital have very similar R_F values and so have antipyrine and vinbarbital as well as phenacetin and pentobarbital. Only subsequent analysis will reveal the true nature of the compound. Antipyrine can further be detected by spraying with Dragendorff's reagent or ferric chloride.

After completion of the run, the papers are air-dried, placed on a paper previously treated with a solution of 0.005 % fluorescein in 0.5 % ammonia, and the "spots" located in ultraviolet light ("Mineralite" of wavelength 254 mμ)*. The R_F values for caffeine, antipyrine, and phenacetin are listed in Table II. The "spots" are eluted

TABLE II

SOME PHYSICAL PROPERTIES OF CAFFEINE, ANTIPYRINE, AND PHENACETIN

(a) Stands for acid pH, (b) for alkaline pH, and (n) for neutral pH

	Caffeine	*Antipyrine*	*Phenacetin*
R_F (chloroform)	0.95	0.95	0.95
R_F (ether)	0.41	0.55	0.90
R_F (*n*-butyl ether)*	0.09	0.13	0.75
Ultraviolet maxima (mμ)**	272 (b) 270 (a) *E* 420 —	244 (b and n) 270 (b and n) 236 (a) *E* 488 *E* 480 —	250 (b, n and a) *E* 822
Ultraviolet minima (mμ)	244 (b, n and a)	256 (b and n)	—
Infrared maxima (cm^{-1})***	1703, 1660, 1548, 1484, 1238, 1023, 970, 738	1685, 1598, 1494, 1393, 1332, 1134	3270, 1658, 1553, 1511, 1482, 1243, 1043, 834
Melting point	238° (subl.)	111–113°	134–135°

* 5 hours running time.
** E = extinction coefficient for 1 % solution and 1 cm lightpath.
*** Intense bands underlined.

from the paper and assayed spectrophotometrically. Paper blanks of similar size are treated in the same way. The eluates from the "spots" are evaporated and dissolved in ethanol–0.5 *M* ammonia (3:1). The spectra of the eluted substances are read both before and after acidification with hydrochloric acid, using the solution of the paper extracts as a blank. The pertinent absorption maxima and minima in the ultraviolet region are listed in Table II. The yields are very satisfactory. When 0.50 mg each of caffeine, antipyrine, and phenacetin were added to 30 g of human liver and the above

* Details of the analytical procedure for barbiturates will be published elsewhere[3].

procedure carried out, over-all yields of 82.7 % and 83.9 % for caffeine, 79.6 % and 79.8 % for antipyrine, and finally 97.3 % and 99.6 % for phenacetin were obtained.

In actual toxicological cases, especially when kidney and urine are extracted, varying amounts of metabolites are found on the chromatograms along with the unaltered compounds[4, 5]. In these cases additional criteria such as infrared spectra and melting point determination are needed for final identification. Filter paper itself contains so much chloroform-soluble foreign material, that the compounds must be subjected to sublimation before satisfactory melting points or infrared spectra can be taken.

Infrared spectra are obtained in the following way. The eluted material containing 10–50 μg of the compound is transferred to a microbeaker, covered with a thin potassium bromide disk (0.5 inch diameter) and placed on the stage of a Kofler block-equipped microscope. The material is sublimated upon the potassium bromide disk and analyzed in the reflecting microscope of a Hilger H 800 infrared spectrophotometer.

Out of about 500 autopsy cases investigated in the past two years, antipyrine was found in 21 cases, phenacetin in 20 cases, and caffeine in 53 cases. About half of the analyses were carried out on a quantitative basis, and up to 8.0 mg antipyrine, 4.0 mg phenacetin, and 1.6 mg caffeine were found per 100 g of tissue. In cases where coffee had been consumed some caffeine is usually found, and sometimes enough theobromine is present to be detected on the chromatogram. With ether as the mobile phase theobromine stays close to the origin, while with chloroform the R_F value is about 0.22.

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SUMMARY

Separation of caffeine, antipyrine and phenacetin from human tissue by paper chromatography is described. The compounds are eluted from the paper and identified by their ultraviolet spectra and by infrared micro spectrophotometry. Methods for quantitative determination are given.

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