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Research Articles

Infrared Analysis of Pharmaceuticals III

Identification and Determination of Adrenocortical Steroids, Barbiturates, and Sulfonamides from Paper Chromatograms

By ALMA L. HAYDEN

The adrenocortical steroids: cortisone, hydrocortisone, and 17-hydroxy-11-desoxycorticosterone, have been identified and estimated from paper chromatograms in amounts between 50 mcg. and 1.5 mg. Recoveries of 90 to 105% of these steroids were obtained on the basis of infrared spectrophotometric determinations. Analyses were made of commercial tablets which contained (*a*) hydrocortisone and 17-hydroxy-11-desoxycorticosterone, or (*b*) the sodium salts of phenobarbital, butabarbital, and pentobarbital. The results agreed within 3.8% with those published earlier (9), or with the declared amounts. In addition, the identification and estimation of sulfanilamide in a sulfacetamide powder were achieved by these methods.

PAPER CHROMATOGRAPHY allows the rapid separation of microgram quantities of compounds. However, the coincidence of R_f and mobility values for a standard and a sample does not afford positive identification in all cases. The value of this separation technique is greatly enhanced when it is combined with other identification methods.

In addition to other applications, paper chromatography and infrared spectroscopy have been combined in the investigation of steroids in human placenta (1). Recently, the use of these

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methods in studies of triamcinolone and related steroids (2), and of caffeine, antipyrine, and phenacetin from human tissue (3) has been reported. Techniques have been published (4–6) whereby the spectra of 100 mcg. and less of some pure crystalline compounds in potassium bromide have been obtained. However, impurities in filter paper and solvents have made the analyses of microgram quantities from paper chromatograms extremely difficult. The instability of some compounds and solvents to evaporation techniques has magnified this problem.

In the present study, mixtures of cortisone and hydrocortisone, in amounts between 50 mcg. and 1.5 mg., were separated on washed Whatman No. 1 filter paper with a chloroform-formamide solvent system (7). After extraction from the chromatograms, the steroids were identified by their infrared spectra in potassium bromide plates and disks. The spectra of samples in amounts less than 100 mcg. were obtained using corresponding blanks in the reference beam and the Perkin-Elmer ordinate scale expander (8). The spectra of samples in amounts greater than 180 mcg. were obtained without compensation or spectrum expansion.

The combined method was applied to commercial tablets which contained hydrocortisone and 17-hydroxy-11-desoxycorticosterone, or pentaerythritol tetranitrate and the sodium salts of phenobarbital, butabarbital, and pentobarbital. The results of the analyses agreed within 3.8% with those reported previously (9), or with the declared amounts. In another application, a commercial sample of sulfacetamide was found to contain about 17% sulfanilamide.

EXPERIMENTAL

Preliminary Infrared Study of the Corticosteroids.—A preliminary study was made to determine the practical amounts of pure cortisone, hydrocortisone, and 17-hydroxy-11-desoxycorticosterone which could be identified by infrared spectroscopy. Dried residues of the steroids, from chloroform and from methanol solutions (0.50 mg. or 1.00 mg. per ml.), in amounts of 50 mcg. to 1.0 mg., were investigated. Amounts of 250 mcg. and above were hand-ground with 200.0 mg. of dry potassium bromide (Harshaw, infrared quality, 200/325 mesh) for exactly 2 minutes. Amounts of 50 to 250 mcg. were hand-ground with 40.0 mg. potassium bromide. The mixtures were dried at 105° for 30 minutes and cooled to room temperature.

A Beckman evacuable die, 0.5 inch in diameter, was used to press the mixtures. A force of 20,000 or 25,000 pounds was applied during evacuation. The 200-mg. loads were pressed into disks 12.7 mm. in diameter. Portions of the 40-mg. loads were pressed into 3 × 10 mm. plates in pressed paper or potassium bromide peripheries (10–12) 12.7 mm. in diameter. Approximately 30 to 35 mg. was needed to fill the 3 × 10 mm. window. The disks and plates were heated at 105° for 15 minutes, and were cooled to room temperature in a desiccator.

The infrared spectra were obtained on a Perkin-Elmer model 21 double-beam spectrophotometer which was equipped with sodium chloride optics, a microcell adapter, and an ordinate scale expander (8). The sample plates and disks were inserted in the sample beam with a holder which fitted the microcell adapter. An optical wedge was placed in the reference beam to compensate for opacity of the pressed paper peripheries. For amounts of 180 mcg. and above of the steroids, spectra of the region between 2 and 15 μ were obtained using the normal (1×) qualitative instrument settings. The spectra of amounts less than 180 mcg. were weak (less than 20% absorption) and poorly defined under the 1× conditions. Therefore, the ordinate scale expander (8) was employed to magnify electrically the pen movement, relative to the optical wedge movement, to five times (5×) its normal (1×) movement. The resulting spectra were similar to, but less defined than, those of amounts greater than 180 mcg.

Spectra of pure compounds which absorb less than 10% or 5% of the incident radiation may be expanded 10 and 20 times, respectively. However, interference from trace impurities in paper chromatogram extracts and poorly resolved spectra limit the usefulness of these expansions.

Paper Chromatography of the Corticosteroids.—Just prior to use, Whatman No. 1 sheets (8 × 8 in.) were eluted, continuously, with methanol for at least 4 hours in an ascending chromatograph tank (13). The sheets were air-dried for at least 30 minutes and were viewed in ultraviolet light (2453Å.). The strongly fluorescent solvent-front area (about 1 cm. deep) along the top edge of the sheet was discarded. The starting line was ruled on the side of each sheet 3 cm. from the edge opposite the solvent-front area. The sheets were used within 24 hours after washing.

About 200 ml. of chloroform (U.S.P. or A.C.S. specifications) was equilibrated with 50 ml. of redistilled formamide for at least 30 minutes. Chromatography tanks were lined with blotter paper and were equilibrated with the chloroform phase for 30 minutes prior to chromatography (13). A solution of the equilibrated formamide in methanol (30:70) was used to impregnate the washed sheets. Excess immobile phase was removed by placing the impregnated sheets between layers of paper towels.

Aliquots of methanolic solutions of cortisone and hydrocortisone (about 1.0 mg./ml.) were applied in mixtures along the starting line of the impregnated sheets. Aliquots containing 50 to 100 mcg. of the steroids were applied, with intermittent air-drying, in spots 1 cm. in diameter. Amounts of 180 mcg. to 1.0 mg. were applied in streaks, 1 cm. × 3 cm. or 1 cm. × 14 cm., in the same manner. Amounts between 100 and 180 mcg. were not studied. After application of the samples, portions of the formamide-methanol solution (0.01 or 0.20 ml.) were added to the spots or streaks to reduce sample adsorption at the starting line (14). The sheets were subjected to ascending chromatography for

about 1 hour. During this time, the solvent traveled approximately 18 cm.

The chromatograms were dried, partially, at room temperature for 30 minutes. The steroid zones were located by their ultraviolet absorbance, using a phosphorescent screen (15), and were outlined on tracing paper. The corresponding zones on the partially dried chromatograms were cut into small rectangular pieces and were suspended in 4.0 ml. of dilute hydrochloric acid (about 0.0025 *N*) in separators. The suspensions were extracted with four 4.0-ml. portions of chloroform, allowing at least 1 minute for each extraction. The extracts of each suspension were washed, successively, with the same 3.0-ml. portion of water, and were filtered through chloroform-washed glass wool into a 50-mm. mullite mortar. The 4.0-ml. extracts of each zone were concentrated, in succession, under nitrogen at about 35°. The extracts were evaporated to dryness only after addition of the final portion of chloroform. The residues were dried in vacuum over phosphorus pentoxide for 30 minutes.

Infrared Spectrophotometry of the Extract Residues.—The dried residues were made into plates or disks as described for the direct standards. For amounts of 180 mcg. and more, the normal (1×) spectra were obtained without blank compensation or ordinate expansion. The spectra of the extracted steroids, in amounts of 100 mcg. and less, were weak and poorly defined, and were affected by small amounts of impurities from the paper and solvents. As a result, compensation with a paper-blank extract and 5 × expansion of the ordinate scale were required to obtain identifiable spectra.

Preliminary studies revealed that compensated spectra may be obtained in two ways. In the instrument used, the microcell-sample position is recessed from the macrocell-sample and reference positions. As a result, the reference beam is larger and less intense per unit area at the sampling position than is the sample beam at the microcell-sample position. A standard disk or plate placed in the latter position absorbs about 1.1 times more strongly than when it is placed in the macro-sample position. Therefore, for compensation, a disk of given effective concentration (concentration × thickness) in the micro-sample position requires a disk 1.1 times as concentrated in the reference position (method I). In method II, since the beams at the macro-sample and reference positions are equivalent, a disk in the macro-sample position is compensated by one of the same effective concentration in the reference position (method II). The holders for the macro-sample and reference positions were rectangular metal plates, 74 × 50 mm., with a central 30 × 7 mm. aperture. Tension clips were soldered on each side of the aperture to position the disks or plates.

Therefore, for compensation, blank paper zones, taken from the same chromatogram and corresponding in weight to the 50 to 100-mcg. steroid zones, were extracted in the manner described above for the steroid zones. The dried residues were ground with 36 mg. (method I) or 40 mg. (method II) of potassium bromide. The mixtures were treated in the same manner as was described for the direct steroids.

In method I, uncompensated scans of the 2 to 15 μ region were made of the sample and of the blank plates in the microcell-sample position under 1 × conditions. At the wavelength of maximum transmission (usually near 5 μ) with the sample in the beam, the blank plate was adjusted in the reference beam to give approximately 95% transmission. The compensated spectrum was obtained between 2 and 15 μ using 1× instrument settings. At the wavelength of maximum transmission, the instrument conditions were changed to those of 5× expansion. The pen was adjusted to 95% transmission with the pen position control. The instrument was reversed to 2 μ without further change. A slow scan of the 2 to 15 μ region was made. In method II, uncompensated scans of the 2 to 15 μ region were made of the sample and of the blank plates in the macro-sample position under 1× conditions. All other operations were identical to those used in method I.

The spectra of the samples were compared with those of disks and plates of the direct standards. The estimated recoveries of cortisone and hydrocortisone were calculated from the normal 1× absorbance at 5.82 μ or 6.02 μ (9) by comparison with the direct standards. The absorptivity coefficients for the direct standards, K_s, were calculated from the equation

$$K_s = (A_s \times H_s)/(C_s \times L_s)$$

In this equation, A_s is the baseline absorbance and H_s is the band width at one-half the height of baseline absorbance of the chosen band, C_s is the ratio of the weight of the standard to the weight of potassium bromide, and L_s is the average thickness of the disk or plate area exposed to the sample beam. The recoveries of the chromatographed steroids were calculated by inserting the appropriate absorptivity coefficient in the following equation

$$\frac{A_u \times H_u}{L_u \times K_s} \times M \times \frac{100}{W} = \text{per cent recovery}$$

A_u and H_u represent the baseline absorbance and half-band width of the sample band, L_u is the average thickness of the exposed sample disk or plate, M is the weight of potassium bromide, and W is the weight of the applied sample.

Analyses of Commercial Products

Hydrocortisone Tablet.—A commercial tablet, declared to contain 20 mg. of hydrocortisone, was suspended in 10 ml. of 0.01 *N* hydrochloric acid. The suspension was extracted with four 25-ml. portions of chloroform. Each chloroform extract was washed with the same 5-ml. portion of 5% sodium carbonate and, finally, with 5 ml. of water. The chloroform was evaporated at about 35° under a stream of nitrogen. The residue was dissolved in absolute methanol and was diluted to 25 ml. in a volumetric flask. A 1.0-ml. aliquot was applied in a streak (1 × 14 cm.) to the starting line of a washed, impregnated sheet of Whatman No. 1 filter paper (8 × 8 inches). The sheet was subjected to ascending chromatography for 50 minutes, and the steroids were detected, extracted, and incorporated into disks (200 mg., 12.7 mm. in diameter) as described earlier.

The spectra were compared, under 1× conditions

between 2 and 15 μ, with those of standard hydrocortisone and 17-hydroxy-11-desoxycorticosterone. Quantitative baseline absorbance and half-band width measurements were made at 5.82 μ for hydrocortisone and at 5.99 μ for 17-hydroxy-11-desoxycorticosterone. The absorptivity coefficient, K_s, for the appropriate standard was inserted in the following equation to obtain the amount of steroid per tablet

$$\frac{A_u \times H_u}{L_u \times K_s} \times M \times \frac{V_t}{V_a} = \text{mg. per tablet}$$

The terms A_u, H_u, L_u, K_s, and M were described earlier. V_a represents the aliquot of the total volume, V_t, taken for analysis.

Tablets of "Triple-barb" (total, 8.0 mg.) and Pentaerythritol Tetranitrate (10.0 mg.).—A sample of powdered tablet mixture containing 8.0 mg. of "Triple-barb" (sodium salts of phenobarbital, butabarbital, and pentobarbital, in equal amounts) was suspended in 5.0 ml. of water in a separator, and 5.0 ml. of 10% sulfuric acid was added. The active ingredients were extracted with chloroform, and the barbiturates were trapped on a Celite-potassium phosphate column as described by Carol (16). The amount of pentaerythritol tetranitrate in a 25.0-ml. aliquot of the chloroform effluent was determined as described (16).

The barbiturates were eluted from the column with chloroform-acetic acid (150 + 15 ml.) and the solvents were evaporated at about 55° under a stream of air. The barbiturate residue was dissolved by warming in three portions of acetone-hydrochloric acid (15 + 0.5 ml.). The solutions were filtered through glass wool, and the combined filtrates were concentrated to about 2 ml. as described above. The concentrate was diluted to 5.0 ml. with acetone.

Immediately, the barbiturates were separated by a modification of the Sabatino procedure (17). A 0.50-ml. aliquot of the barbiturate solution was applied in a streak to washed filter paper. The filter paper was mist-sprayed with 0.5 M sodium carbonate and was subjected to continuous ascending chromatography (13) for 4 hours with ethylene chloride as the mobile solvent.

The chromatogram was air-dried, and the three ultraviolet absorbing zones were suspended in 5.0-ml. portions of 5% hydrochloric acid. The suspensions were extracted and the extracts were treated as described for the steroids. The extract residue of each barbiturate was dissolved in 0.50 ml. chloroform; 3.0 ml. n-heptane was added, and the solvents were evaporated under nitrogen at about 35°. Crystalline samples of the standards (about 250.0 mcg.) were treated in the same manner. Potassium bromide disks (200 mg., 12.7 mm. in diameter) were prepared of the solid residues. The disks of phenobarbital and butabarbital were heated at 105° for 30 minutes. Because of difficulty in controlling the polymorphic forms, the disks of pentobarbital were heated above the melting point of pentobarbital at 143° for 30 minutes. The disks were cooled to room temperature, and the uncompensated quantitative spectra were obtained between 5.3 and 6.6 μ using normal 1× instrument conditions.

From the baseline absorbances A_u of the bands near 5.65 and 5.85 μ, and the average thicknesses L_u of the disks, the amounts of the barbiturate sodium salts per tablet were calculated from the following equations

$$A_s/C_s \times L_s = K_s$$

$$\frac{A_u}{L_u \times K_s} \times M \times \frac{V_t}{V_a} \times \frac{T}{W} \times Z = \text{mg./tablet}$$

The terms A_u, L_u, M, V_t, and V_a were described earlier. T and W represent the average weight per tablet and the sample weight, respectively. Z is the ratio of the molecular weight of the sodium salt to that of the barbituric acid.

The identities of the chromatographed barbiturates were proved by comparing their spectra (1×) in the 2 to 15 μ region with those of the direct standards.

Mixture of Sulfacetamide and Sulfanilamide.—A 100.0-mg. sample of the commercial powder was dissolved in 1.0 ml. of ammonium hydroxide and the solution was diluted to 10.0 ml. with water. A methanol-washed filter paper sheet was impregnated with formamide-acetone (30:70) as described by Maienthal and co-workers (18). A 0.100-ml. aliquot of the sample solution was applied in a streak (1 × 14 cm.) as described previously. The filter paper was subjected to continuous ascending chromatography for 3 hours with chloroform-tertiary butyl alcohol (100 + 6 ml.) as the mobile solvent.

The sheet was air-dried in the dark for about 1 hour and was viewed, briefly, under ultraviolet light. The ultraviolet-absorbing sample zones were outlined, cut into small rectangles, and extracted by warming with four 10.0-ml. portions of acetone for 15 minutes. Each extract was filtered through glass wool, the filtrates were evaporated to near dryness, and the concentrate was transferred to a 50-mm. mortar with several small portions of acetone. The sample solutions and 2.0-ml. aliquots of standard sulfacetamide and of sulfanilamide in acetone (0.200 mg./ml.) were evaporated to dryness as described previously. The residues were dried at 80° in a vacuum oven for 1 hour. The cooled residues were triturated with 2 ml. of heptane for about 10 minutes and the solvent was evaporated at 35° under nitrogen.

Potassium bromide disks (200.0 mg., 12.7 mm. in diameter) were prepared of the sulfacetamide standard, and sample residues, and of the sulfanilamide standard. A potassium bromide rectangle (3 × 10 mm.) in pressed paper periphery was prepared of the sulfanilamide paper chromatography residue. Quantitative spectra were obtained of the heated standard and sample disks and plate between 8.0 and 9.75 μ. Measurements were made of the baseline absorbance and the band widths at two-thirds the height of absorbance of the 9.16 μ band. The per cent of each compound in the commercial mixture was calculated using the equation

$$\frac{A_u \times H_u}{L_u \times K_s} \times M \times \frac{V_t}{V_a} \times \frac{100}{W} = \text{per cent recovery}$$

The identities of the extract residues were proved by comparison of the spectra (1×) of their disks or plates in the 2–15 μ region with those of the direct standards.

RESULTS AND DISCUSSION

Table I shows that cortisone and hydrocortisone are well separated even in 1:8 and 11:1 mixtures. Except for slightly lower values for the 50-mcg. zones, the mobilities were reproducible.

In Fig. 1 are spectra of chromatographed cortisone (II, 180 mcg.) and hydrocortisone (IV, 1.46 mg.) obtained under normal 1× conditions. The uncompensated spectra are identical to those of the corresponding direct standards (I, 180 mcg.; and III, 470 mcg.) and to published spectra (12). Figure 2 gives expanded (5×) spectra of chromatographed cortisone (I, 50 mcg.) and hydrocortisone (II, 100 mcg.) compensated with corresponding blanks. In general, the expanded spectra are similar to but less resolved than those obtained under normal operating conditions. The background absorbance in the C—H stretching and bending regions is due to uncompensated impurities from the paper and solvent.

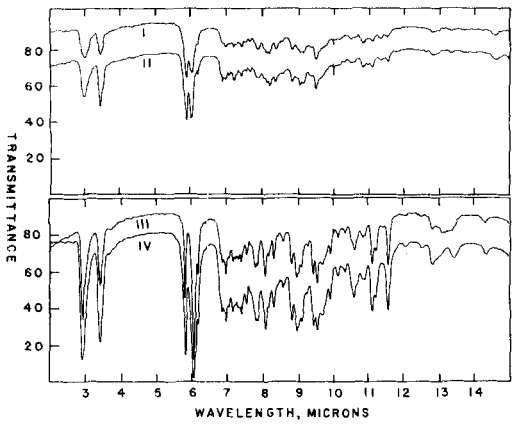

Fig. 1.—Infrared spectra of chromatographed cortisone (II) and hydrocortisone (IV) and the corresponding direct standards (I and III).

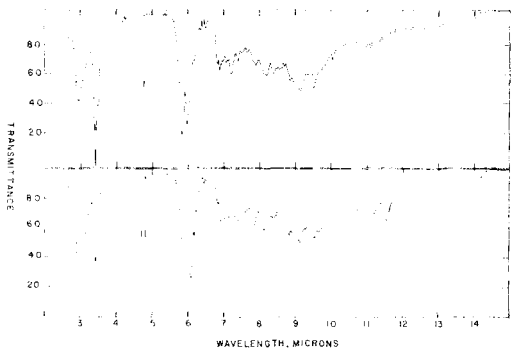

Fig. 2.—Expanded (5×) infrared spectra of chromatographed cortisone (I, 50 mcg.) and hydrocortisone (II, 100 mcg.)

The background absorbance was reduced by using freshly washed paper, by extracting chromatograms while they were still damp, by using a minimum volume of chloroform, and by evaporating the extracts immediately under nitrogen. Evaporation of washed commercial chloroform revealed a negligible residue. Where significant residues are obtained, the commercial chloroform should be redistilled or otherwise purified. The stopcocks were wetted with water only; other lubricants were avoided. All containers were rinsed with a suitable solvent before use. Background absorbance was greatly increased if ethyl alcohol was used as a rinse or as a stabilizer of the chloroform extracts. When the washed sheets were allowed to stand for days or weeks before use, the absorbance of paper blanks was increased.

Some estimations of the recoveries of the chromatographed cortisone and hydrocortisone were made by comparisons with the direct standards. The results of these analyses are given in Table II. Recoveries of 90 to 105% of the applied steroids were obtained. The lowest recoveries were obtained with amounts of 50 to 100 mcg. For best qualitative and quantitative results, it is recommended that amounts of 180 mcg. or greater of the steroids be used.

Paper chromatography of the hydrocortisone tablet extract revealed two ultraviolet absorbing zones. The more polar zone (2.0–4.4 cm.) corresponded to hydrocortisone in mobility. The less polar zone (9.3–11.7 cm.) had a mobility similar to 17-hydroxy-11-desoxycorticosterone. The infrared spectra of the extracted materials were identical to the standards. Quantitative measurements were made of the chromatographed steroids and the corresponding standards. Hydrocortisone and 17-hydroxy-11-desoxycorticosterone were present in the tablets in amounts of 46.1 and 53.9%, respectively. Previous analyses of this tablet by column chromatography and infrared spectrophotometry (9) revealed 47.5% hydrocortisone and 52.5% 17-hydroxy-11-desoxycorticosterone.

The paper chromatogram of the barbiturate fraction revealed three ultraviolet absorbing zones with mobilities similar to those of phenobarbital (2.0–3.8 cm.), butabarbital (4.1–6.1 cm.), and pentobarbital (6.4–8.8 cm.). The mobilities were dependent on the amount of immobile phase used to impregnate the paper. The infrared spectra of the paper chromatogram extracts were identical to those of the corresponding standards. The recoveries (Table III) agreed within 3.8% with the declared amounts.

The paper chromatogram of the sulfacetamide sample revealed two ultraviolet zones which corresponded in mobility to those of standard sulfacetamide (1.6–3.4 cm.) and sulfanilamide (0.0–1.6 cm.). The identities of these components were confirmed by comparing their infrared spectra with those of standard sulfacetamide and sulfanilamide. Sulfacetamide and sulfanilamide constituted 70.1 and 17.0%, respectively, of the total powder (Table III).

From these results, the general usefulness of the combined method is apparent. To the rapid and efficient paper chromatographic separation is added the specificity of the infrared spectra. The method should be adaptable to a variety of compounds. With modifications, such as the use of guide strips for color development, the study of ultraviolet transparent compounds could be made.

It is possible that improved qualitative and quan-

TABLE I.—MOBILITIES OF CORTISONE AND HYDROCORTISONE[a]

Sample No.	Hydrocortisone		Cortisone	
	Amount, mcg.	Mobility, cm.	Amount, mcg.	Mobility, cm.
1	50	1.8–4.4	50	5.6– 8.8
2	100	2.8–5.1	100	7.1–10.1
3	200	2.9–5.1	200	7.4–10.2
4	500	2.2–4.4	500	6.4– 9.3
5	1,460	2.1–5.1	186	7.7–10.3
6	92	2.6–5.0	1,000	7.0–11.4

[a] The mobility values give the distances of the leading and following edges of the zones from the starting line.

TABLE II.—RECOVERIES FROM CHROMATOGRAMS OF MIXTURES

Sample No.	Cortisone			Hydrocortisone		
	Applied, mcg.	Found, mcg.	Recovery, %	Applied, mcg.	Found, mcg.	Recovery, %
1	50	46	92.0	...	...	...
2	100	90	90.0	100	93.7	93.7
3	186	189	101.6	1,460	1,436.0	98.4
4	1,000	1,050	105.0	100	99.6	99.6

TABLE III.—RECOVERIES FROM COMMERCIAL PREPARATIONS

Sample	Active Ingredients	Declared, mg./Sample	Found	
			mg.	%
1	Hydrocortisone	20.00	9.22	46.1
	17-Hydroxy-11-desoxycorticosterone	0.00	10.78	53.9
2[a]	Phenobarbital sodium	2.67	2.77	103.7
	Butabarbital sodium	2.67	2.57	96.2
	Pentobarbital sodium	2.67	2.77	103.7
3	Sulfacetamide	100.00	70.10	70.1
	Sulfanilamide	0.00	17.00	17.0

[a] Pentaerythritol tetranitrate was present as 101.15% of declared amount.

titative results can be obtained on amounts less than 100 mcg. by using especially purified solvents and paper. Additional purification steps would be required in the analysis of compounds from biological media. It is anticipated that this method will be useful in the analyses of compounds from varied origins. Instruments equipped with beam condensers can be used if the aforementioned differences in beam sizes are considered.

SUMMARY

Cortisone and hydrocortisone were extracted from paper chromatograms and identified by their infrared spectra. The range of recoveries of amounts of 50 mcg. to 1.5 mg. was 90 to 105%. Analyses of commercial preparations, which contained corticosteroids, barbiturates, or sulfonamides, were achieved by paper chromatography and infrared spectrophotometry.

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